

(19)



Europäisches Patentamt

European Patent Office

Office européen des brevets



(11)

EP 0 845 270 A1

(12)

EUROPEAN PATENT APPLICATION

published in accordance with Art. 158(3) EPC

(43) Date of publication:
03.06.1998 Bulletin 1998/23

(51) Int. Cl.⁵: A61K 39/08, C12N 15/00,
C12N 15/31, C12P 21/02
// C07K14/33

(21) Application number: 97907448.1

(22) Date of filing: 24.03.1997

(86) International application number:
PCT/JP97/00976

(87) International publication number:
WO 97/35612 (02.10.1997 Gazette 1997/42)

(84) Designated Contracting States:
BE CH DE DK FR GB IT LI NL

(30) Priority: 23.03.1996 JP 106053/96

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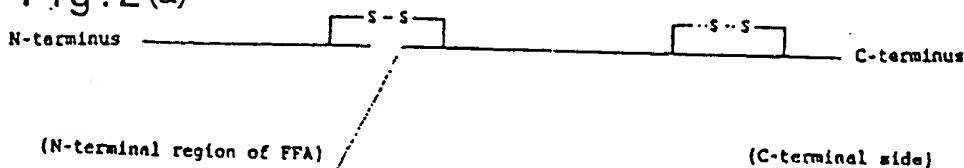
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(54) FUNCTIONAL FRAGMENT ANTIGEN OF TETANUS TOXIN AND TETANUS VACCINE

(57) A functional fragment antigen of tetanus toxin characterized in that the antigen comprises a fragment which is substantially the same as at least one type of the fragments obtained by cleaving at least one of the peptide bonds formed between the amino acid residues in the partial amino acid sequence present between the two cysteine residues participating in the disulfide bridge present on the N-terminal side in the full-length amino acid sequence of a full-length tetanus toxin molecule, also cleaving the disulfide bridge itself, and further cleaving the non-covalent bonds between the amino acid residues that constitute the toxin molecule peptide, that it has a molecular weight of 90,000 to

110,000 as determined by SDS-polyacrylamide gel electrophoresis and an isoelectric point of 7.25±0.5 as determined by isoelectric electrophoresis, and that it has an immunogenicity substantially equal to that of a full-length tetanus toxin molecule. This antigen keeps an immunogenicity as a tetanus vaccine antigen and is remarkably reduced in side effects. The invention also provides a process for the mass production of the functional fragment antigen, a vaccine containing this antigen, and a mixed vaccine comprising the above vaccine and a different vaccine.

Fig. 2(a)



K I I P P T H N I R E N L Y N R T A S L T D L G G E L C I K - (1)
I I P P T H N I R E N L Y N R T A S L T D L G G E L C I K - (2)
E N L Y N R T A S L T D L G G E L C I K - (3)
N L Y N R T A S L T D L G G E L C I K - (4)
N R T A S L T D L G G E L C I K - (5)
T A S L T D L G G E L C I K - (6)
S L T D L G G E L C I K - (7)
G G E L C I K - (8)

Description

BACKGROUND OF THE INVENTION5 Technical Field

The present invention relates to a tetanus toxin functional fragment antigen and a tetanus vaccine comprising the same. More particularly, the present invention is concerned with a specific tetanus toxin functional fragment antigen which is extremely useful as an antigen for a tetanus vaccine since the functional fragment antigen is advantageous not only in that it is extremely excellent with respect to the diminution of side effects when used as an antigen, as compared to the current tetanus vaccine comprising as an antigen a whole tetanus toxin toxoid, but also in that it has an immunopotency which is substantially the same as that of the whole tetanus toxin toxoid. The present invention is also concerned with a very safe and effective tetanus vaccine (tetanus toxoid) comprising the tetanus toxin functional fragment antigen as an active component, a combined vaccine comprising the tetanus vaccine and at least one vaccine other than the tetanus vaccine, and methods for producing the fragment antigen and vaccines.

Prior Art

As is well known, tetanus is an infectious disease with extremely high mortality which produces serious symptoms, such as opisthotonos and dyspnea. Tetanus bacilli are widely distributed in the environments, and their spores are commonly found in soil, feces of animals, and the like. Therefore, every individual is exposed to the danger of tetanus infection from various types of traumas, such as punctured wounds and crushed wounds. Moreover, when an individual is infected with tetanus bacilli, conventional chemotherapies using antibiotics, muscle relaxants and the like cannot grossly change the mortality, whether tetanus patients are elderly or young. In developed countries, most deaths from tetanus have recently occurred among the elderly patients who escaped from vaccination against tetanus in their babyhood.

Further, even when an individual receives tetanus vaccination in baby- or child-hood for basal immunization and receives a booster, the immunity remaining in adulthood is not sufficient for preventing tetanus infection when the individual suffers unexpected injury in earthquakes, fires, traffic accidents or the like. Therefore, it is important for adults of the ages above ca. 40, especially elderly persons, to receive personally a booster injection in order to ensure protection against tetanus.

In developed countries, an increase in the number of intrahospital childbirths, and improvements in living environments and sanitation, an improvement in the quality of emergency medical care with respect to the provision of toxoids and antitoxins, and compulsory vaccinations for younger people, have reduced the number of tetanus patients to 1/30 of that of half a century ago. Furthermore, tetanus is a non-epidemic disease and is not transmitted from person to person. Therefore, the importance of the prevention of this disease tends to be overlooked. However, even today, the number of tetanus deaths in the world is estimated to be about 1 million per year, including mostly neonatal tetanus deaths which are prevailing in developing countries. In addition, due to widespread drug abuse, the number of tetanus patients infected through contaminated injection needles is also increasing recently.

Under these circumstances, tetanus is now recognized as a disease to be prevented by vaccination, rather than to be treated, and preventive measures against tetanus are being actively undertaken. For example, in the Expanded Program of Immunization (EPI) of the World Health Organization (WHO), vaccination against tetanus is being adopted as one of the most important tasks, and the vaccination program is being promoted. The "International Conference on Tetanus", one of whose goals is to eradicate the tetanus disease has been held about every three years in various countries since 1963.

As evident from the above, tetanus is a disease caused by a ubiquitous bacteria whose spores are impossible to eradicate from the earth, and it is not an exaggeration to say that vaccination against tetanus is the only way to reduce the death of human beings due to tetanus, irrespective of age and sex, to zero, and that the vaccination is essential for

tant vaccines. However, the current tetanus toxoid preparations have various problems to be solved. For example, the tetanus toxoid has disadvantages in that there are various adverse side effects, that the product quality is uneven among different manufacturers, that the retention of immunity is limited to only approximately 5 to 10 years and, therefore, repeated vaccinations are necessary to keep the antitoxin level sufficient to prevent tetanus infection. Thus, the conventional tetanus toxoid has problems to be solved with respect to safety, control of quality, retention of immunity, and ease, labor saving and economy in administration. Therefore, for promoting the use of the tetanus vaccine, a large number of problems need to be solved mainly from a viewpoint of mass-production of high quality tetanus vaccine.

Hereinbelow, prior art is discussed in connection with the primary object of the present invention, which is to provide a tetanus toxin antigen, which is not only extremely excellent with respect to the diminution of adverse side effects when used as a vaccine, but also exhibits high immunopotency, thus solving the above-mentioned problems accompanying the prior art.

Various adverse side effects are known to accompany the use of conventional tetanus toxoid vaccines. Various adverse side effects, such as local reactions at injection sites (e.g., erythema, tenderness, swelling, edema and sterile abscess), systemic fever; and, although rare, allergy (e.g., local anaphylaxis, anaphylactic shock, serum sickness-like type III hypersensitivity and delayed hypersensitivity) and serious generalized reactions (e.g., peripheral neuropathy, lymphadenopathy, brachial plexus neuropathy, Guillain-Barret syndrome and acute transverse myelitis) have been reported (see "Vaccine", 2nd edition, edited by S.A. Plotkin and E.A. Mortimer, pp. 75-77, W. B. Saunders Company, 1994; "Mandell, Douglas and Bennett's Principles and Practice of Infectious Diseases", 4th edition, edited by G. L. Mandell et al., p. 2781, Churchill Livingstone & Son, Inc., 1995; Journal of the American Medical Association, 264(18), p. 2448, 1990 and 271(20), p. 1629, 1994; and Lancet, 339, pp. 1111-1112, 2 May, 1992).

Various attempts to reduce or remove these adverse side effects of the tetanus toxoid vaccine have been made. For example, development of a method for obtaining highly purified toxoid, use of modified or new adjuvants, and individual use of the fragments A, B and C (which are subunits of the tetanus toxin and which are explained below) as an active component for a vaccine have been proposed. Of these attempts, with respect to the techniques of using a tetanus toxin fragment, as examples of tetanus toxin fragments used in these techniques, there can be mentioned fragment C prepared by digesting the tetanus toxin with trypsin and/or papain (see Unexamined Japanese Patent Application Laid-Open Specification Nos. 50-71820, 51-82719 and 52-83928), fragment A-B prepared by digesting the tetanus toxin with papain (see Unexamined Japanese Patent Application Laid-Open Specification No. 53-26319), an antigen obtained by expressing a gene coding for fragment C in *E. coli*, yeast or salmonella (see Unexamined Japanese Patent Application Laid-Open Specification No. 3-285681, Japanese Patent Application prior-to-examination Publication (Kohyo) No. 4-506005, International Application Publication Nos. WO 90/15871 and WO 94/03615, and EP-A-O 209 281), and a synthesized epitope of fragment C (see International Application Publication No. WO 94/00484). However, none of these conventional tetanus toxin fragment vaccines have been put into practical use because all of these tetanus toxin fragment vaccines have low antigenicity and immunopotency, as compared to those of the conventional tetanus toxoid comprising the toxoid of the whole tetanus toxin molecule. Meanwhile, cloning of the tetanus toxin gene, and

tency which is substantially the same as that of a whole tetanus toxin toxoid.

It is another object of the present invention to provide a tetanus vaccine which is extremely excellent with respect to the diminution of adverse side effects of the current vaccines, and has an immunopotency which is substantially the same as that of the conventional whole tetanus toxoid vaccine.

A further object of the present invention is to provide a method for producing the above-mentioned tetanus vaccine.

Still a further object of the present invention is to provide a method for producing the above-mentioned functional fragment antigen (FFA) as a tetanus vaccine antigen.

The foregoing and other objects, features and advantages of the present invention will be apparent to those skilled in the art from the following detailed description and appended claims taken in connection with the accompanying sequence listing and drawings.

BRIEF DESCRIPTION OF THE SEQUENCE LISTING

SEQ ID NO. 1 is one form of the entire amino acid sequence of the whole tetanus toxin molecule used in the present invention.

BRIEF DESCRIPTION OF THE DRAWINGS

In the accompanying drawings:

Fig. 1(a) is a diagrammatic view of the structure of the whole tetanus toxin molecule used in the present invention;

Fig. 1(b) is a diagrammatic view of a nicked form of the whole tetanus toxin molecule;

Fig. 1(c) shows a tripartite [A-B · C] model of the whole tetanus toxin molecule;

Fig. 2(a) is a diagrammatic view showing a variety of the N-terminal amino acid sequences of the tetanus toxin functional fragment antigen; and

Fig. 2(b) is a diagrammatic view of a structural model of the whole tetanus toxin molecule.

Description of Reference Characters

S -- S: disulfide bridge

* : nick

----- : non-covalent bond

In the one-letter representation system for representing the amino acid residues of an amino acid sequence, the letters respectively represent the following amino acid residues:

A	Alanine	C	Cysteine	D	Aspartic acid
E	Glutamic acid	F	Phenylalanine	G	Glycine
H	Histidine	I	Isoleucine	K	Lysine
L	Leucine	M	Methionine	N	Asparagine
P	Proline	Q	Glutamine	R	Arginine
S	Serine	T	Threonine	V	Valine
W	Tryptophan	Y	Tyrosine		

DETAILED DESCRIPTION OF THE INVENTION

According to the present invention, there is provided a tetanus toxin functional fragment antigen, comprising at least one fragment which is substantially the same as that obtained by a process comprising the steps of splitting at least one peptide bond selected from peptide bonds individually connecting mutually adjacent amino acid residues in a partial amino acid sequence between two cysteine residues participating in forming a disulfide bridge present in the N-terminal of the entire amino acid sequence of a whole tetanus toxin molecule, splitting the disulfide bridge, and splitting non-covalent bonds [as indicated in Fig. 2(b)] between groups on the tetanus toxin molecule;

the tetanus toxin functional fragment antigen having:

- (a) a molecular weight of from 90,000 to 110,000 as measured by an SDS-polyacrylamide gel electrophoresis method;
- (b) an isoelectric point of 7.25 ± 0.5 as measured by an isoelectric focusing method; and
- (c) an immunopotency which is substantially the same as that of a whole tetanus toxin toxoid.

For easy understanding of the present invention, the essential features and various preferred embodiments of the present invention are enumerated below.

1. A tetanus toxin functional fragment antigen, comprising at least one fragment which is substantially the same as that obtained by a process comprising the steps of splitting at least one peptide bond selected from peptide bonds individually connecting mutually adjacent amino acid residues in a partial amino acid sequence between two cysteine residues participating in forming a disulfide bridge present in the N-terminal of the entire amino acid sequence of a whole tetanus toxin molecule, splitting the disulfide bridge, and splitting non-covalent bonds between groups on the tetanus toxin molecule;

the tetanus toxin functional fragment antigen having:

- (a) a molecular weight of from 90,000 to 110,000 as measured by an SDS-polyacrylamide gel electrophoresis method;
- (b) an isoelectric point of 7.25 ± 0.5 as measured by an isoelectric focusing method; and
- (c) an immunopotency which is substantially the same as that of a whole tetanus toxin toxoid.

2. The tetanus toxin functional fragment antigen according to item 1 above, wherein each of the at least one fragment independently has an N-terminal amino acid sequence selected from the group consisting of the following amino acid sequences (1) to (8):

- (1) KIIPTNNIRENLYNRTASLTDLGGELCIK,
- (2) IIPPTNNIRENLYNRTASLTDLGGELCIK,
- (3) ENLYNRTASLTDLGGELCIK,
- (4) NLYNRTASLTDLGGELCIK,
- (5) NRTASLTDLGGELCIK,
- (6) TASLTDLGGELCIK,
- (7) SLTDLGGELCIK, and
- (8) GGELCIK .

3. The tetanus toxin functional fragment antigen according to item 1 or 2 above, which is stabilized with a fixative.

4. A tetanus vaccine comprising, as an active component, the tetanus toxin functional fragment antigen of any one of items 1 to 3 above in an effective immunogenic amount.

5. A combined vaccine comprising, as one of a plurality of active components, the tetanus toxin functional fragment antigen of any one of items 1 to 3 above in an effective immunogenic amount.

6. A method for producing a tetanus vaccine, which comprises stabilizing a tetanus toxin functional fragment antigen with a fixative,

the tetanus toxin functional fragment antigen comprising at least one fragment which is substantially the same as that obtained by a process comprising the steps of collecting and purifying an extracellular tetanus toxin from a culture filtrate of *Clostridium tetani* to obtain an extracellular tetanus toxin molecule, splitting a disulfide bridge present in the N-terminal of the entire amino acid sequence of the extracellular tetanus toxin molecule, and splitting non-covalent bonds between groups on the extracellular tetanus toxin molecule;

the tetanus toxin functional fragment antigen having:

- (a) a molecular weight of from 90,000 to 110,000 as measured by an SDS-polyacrylamide gel electrophoresis method;
- (b) an isoelectric point of 7.25 ± 0.5 as measured by an isoelectric focusing method; and
- (c) an immunopotency which is substantially the same as that of a whole tetanus toxin toxoid.

7. A method for producing a tetanus toxin functional fragment antigen, comprising:

ligating a DNA coding for the tetanus toxin functional fragment antigen of item 1 or 2 above to a vector; transforming host cells, exclusive of *Clostridium tetani*, with the vector; and expressing the DNA coding for the tetanus toxin functional fragment antigen.

Hereinbelow, the present invention is described in detail.

In the present invention, Ala represents an alanine residue, Arg represents an arginine residue, Asn represents an asparagine residue, Asp represents an aspartic acid residue, Cys represents a cysteine residue, Gln represents a glutamine residue, Glu represents a glutamic acid residue, Gly represents a glycine residue, His represents a histidine residue, Ile represents an isoleucine residue, Leu represents a leucine residue, Lys represents a lysine residue, Met represents a methionine residue, Phe represents a phenylalanine residue, Pro represents a proline residue, Ser represents a serine residue, Thr represents a threonine residue, Trp represents a tryptophan residue, Tyr represents a tyrosine residue and Val represents a valine residue.

As mentioned above, the tetanus toxin functional fragment antigen of the present invention comprises at least one fragment which is substantially the same as that obtained by a process comprising the steps of splitting at least one peptide bond selected from peptide bonds individually connecting mutually adjacent amino acid residues in a partial amino acid sequence between two cysteine residues participating in forming a disulfide bridge present in the N-terminal of the entire amino acid sequence of a whole tetanus toxin molecule, splitting the disulfide bridge, and splitting non-covalent bonds [as indicated in Fig. 2(b)] between groups on the tetanus toxin molecule.

As a preferred embodiment of the tetanus toxin functional fragment antigen of the present invention, there can be mentioned the tetanus toxin functional fragment antigen, wherein each of the above-mentioned at least one fragment independently has an N-terminal amino acid sequence selected from the group consisting of the following amino acid sequences (1) to (8):

(1) KIIPPTNNIRENLYNRTASLTDLGGE LCIK,

(2) IIPPTNNIRENLYNRTASLTDLGGE LCIK,

(3) ENLYNRTASLTDLGGE LCIK,

(4) NLYNRTASLTDLGGE LCIK,

(5) NRTASLTDLGGE LCIK,

(6) TASLTDLGGE LCIK,

(7) SLTDLGGE LCIK, and

(8) GGELCIK .

Further, the tetanus toxin functional fragment antigen of the present invention may be stabilized with a fixative. For further clarification of the essential features of the present invention, the technical features of the present inven-

tion will be described by explaining the development of the present invention.

Isolation of a strain of Clostridium tetani having high toxin-producing ability:

5 In the present invention, a strain of Clostridium tetani having high toxin-producing ability is used. The present inventor isolated a substrain having high toxin-producing ability by single colony isolation from Harvard H47 strain, which is a known C. tetani strain derived from a known C. tetani strain called the Harvard strain [ATCC (American Type Culture Collection) accession No. 10779], and he designated the obtained substrain as "Clostridium tetani Harvard H47 strain Biken substrain" (hereinafter referred to simply as "Biken substrain"). Also, the present inventor found that production
10 of tetanus toxin is under the control of genetic information carried by the plasmid DNA in the C. tetani cell (Biken Journal, 20, 105-115, 1977). Further, by using the culturing method based on the above finding, the present inventor succeeded in mass production of tetanus toxin by culturing the Biken substrain, and high purification of the tetanus toxin.

Thus, in the present invention, it is first necessary that a strain of Clostridium tetani having high toxin-producing ability is selected and used as a seed culture. As a seed culture, a culture of transformant of a microorganism, such as
15 yeast, Escherichia coli, Bacillus subtilis or the like, which is obtained using the below-mentioned DNA coding for FFA, by genetic engineering techniques, can be used.

Mode of formation of and toxic activity of tetanus toxin:

20 In the C. tetani cells, tetanus toxin is first produced in the form of a single polypeptide chain (whole tetanus toxin molecule in a non-nicked, intact form) having a molecular weight of about 150,000 (hereinafter, frequently referred to as "intracellular toxin"). Subsequently, by the autolysis of the cell, the tetanus toxin is released from the cells into the extracellular medium (hereinafter, the toxin released into the extracellular medium is frequently referred to as "extracellular toxin"). When the toxin is released from the cells, at least one bond in the peptide bonds connecting mutually adjacent amino acid residues in the partial amino acid sequence between two cysteine residues participating in forming the
25 disulfide bridge present in the N-terminal of the whole amino acid sequence of the whole tetanus toxin molecule is split by a protease produced by C. tetani, to thereby form at least two polypeptide chains. However, the two polypeptide chains are united to each other by the disulfide bridge present in the N-terminal of the whole tetanus toxin molecule, that is, these polypeptide chains assume a nicked form [see Figs. 1(a) and 1(b); Biochemical and Biophysical
30 Research Communications, 57, 1257-1262, 1974; *ibid.* 68, 668-674, 1976; *ibid.* 77, 268-274, 1977], and further, the two polypeptide chains are also united to each other by non-covalent bonds [see Fig. 2(b)]. Conversion of the tetanus toxin molecule from the intact form into the nicked form enhances the toxin activity of the tetanus toxin several times, and the nicking is essential for eliciting toxic action. Therefore, for saving labor in the preparation of the functional fragment antigen, it is preferred to use, as a starting material, the extra-cellular tetanus toxin, which has already been converted into
35 the nicked form [see Fig. 1(b)].

Subunit structure of tetanus toxin and the mechanism of manifestation of toxicity of tetanus toxin:

As a result of the unique studies of the present inventor, two functionary complementary polypeptide chains,
40 namely, L (light) chain and H (heavy) chain, were obtained from the whole tetanus toxin molecule. That is, the present inventor succeeded in isolating and purifying these fragments (L and H chains), to thereby obtain the L chain and H chain individually, which are native enough to be able to reproduce the toxin activity of the whole tetanus toxin molecule when these L and H chains are reconstituted into the whole tetanus toxin molecule, although each of the individually obtained L chain and H chain is not toxic.

45 In addition, the present inventor also successfully isolated and purified the following three fragments: the C-terminal half of the H chain (fragment C), a fragment (fragment A-B) obtained by removing fragment C from the whole tetanus toxin molecule, and a fragment (fragment B) obtained by separating fragment A (i.e., L chain) from the purified fragment A-B.

Further, after the preparation of the whole tetanus toxin molecule and the above-mentioned fragments A, B, C, A-

nus toxin molecule into the cytoplasm (letter "B" means "Binding"), and fragment A has the role of exhibiting the toxic activity based on the enzyme activity (letter "A" means "Active") (see "8th International Conference on Tetanus", edited by G. Nistco et al., pp. 170-171, Phythagora Press, Rome-Milan, 1989; Infection and Immunity, 57, 3588-3593, 1989; Toxicon, 27, 385-392, 1989; ibid. 28, 737-741, 1990).

Variety of the fragments of tetanus toxin:

Until 1989, there was no consensus on any of the length, molecular weight and nomenclature of each of the fragments of tetanus toxin among researchers in the world, and this situation gave difficulties in exchange of information and discussions on the structures-function relationship of subunits of tetanus toxin among researchers. Therefore, it was desired to establish a common basis for the studies on tetanus toxin by using unitary definitions of fragment models.

In such a situation, the present inventor proposed the above-mentioned tripartite molecular model for the first time. That is, the present inventor pointed out the disadvantages of the absence of a consensus on the lengths, molecular weights and nomenclatures of the tetanus toxin fragments, and the present inventor emphasized the necessity of unitary definitions of fragments and proposed the above models [see the above-mentioned "Botulinum Neurotoxin and Tetanus toxin", edited by L. L. Simpson, pp. 69-92 (Chapter 4)].

It is believed that the reason why a variety of fragments are obtained from the whole tetanus toxin molecule resides not only in the genetic differences of seed strains of *C. tetani* used by different researchers, but also in that there are delicate differences in various operation conditions employed by researchers for obtaining tetanus toxin and fragments thereof, such as the culturing conditions for the seed strain, the autolysis conditions for the cultured cells to obtain the extracellular toxin which has already been converted into a nicked form, and the treating conditions for an extracted intracellular toxin, that is, the conditions for digesting the extracted intracellular toxin by a protease into a nicked form, and the conditions for treating the extracted intracellular toxin with a reducing agent, a denaturing agent, a solubilizing agent or the like, wherein examples of conditions for treating the extracted intracellular toxin include the types of the enzyme and reagents, the treatment temperature, the treatment time, the concentration of the enzyme or reagent, the pH of the treating solution, and the physical conditions for the treatment of the extracted intracellular toxin, i.e., stirring or shaking, or keeping it in a stationary state.

Definition of "FFA" of the present invention:

The functional fragment antigen (FFA) of the present invention is a tetanus toxin functional fragment antigen, comprising at least one fragment which is substantially the same as that obtained by a process comprising the steps of splitting at least one peptide bond selected from peptide bonds individually connecting mutually adjacent amino acid residues in a partial amino acid sequence between two cysteine residues participating in forming a disulfide bridge present in the N-terminal of the entire amino acid sequence of a whole tetanus toxin molecule, splitting the disulfide bridge, and splitting non-covalent bonds [as indicated in Fig. 2(b)] between groups on the tetanus toxin molecule;

the tetanus toxin functional fragment antigen having:

- (a) a molecular weight of from 90,000 to 110,000 as measured by an SDS-polyacrylamide gel electrophoresis method;
- (b) an isoelectric point of 7.25 ± 0.5 as measured by an isoelectric focusing method; and
- (c) an immunopotency which is substantially the same as that of the whole tetanus toxin molecule.

As a result of the research by the present inventor, it has been found that various types of N-terminal amino acid sequences of FFA can be obtained [see Fig. 2(a)]. In the present invention, it is preferred that the tetanus toxin functional fragment antigen has an N-terminal amino acid sequence selected from the group consisting of the eight amino acid sequences shown in Fig. 2(a). Further, the tetanus toxin functional fragment antigen (FFA) of the present invention has an immunopotency which is substantially the same as that of the whole tetanus toxin toxoid. In addition, the tetanus toxin functional fragment antigen (FFA) of the present invention is extremely excellent with respect to the diminution of

challenge toxin of a known LD₅₀ (Median Lethal Dose) described in Example 1(5). The results are analyzed by the score method described in Reference Example 15.

Thus, the present invention also provides a single-antigen tetanus vaccine comprising FFA as an active component, such as a plain preparation, an adsorbed preparation or a lyophilized preparation, and a combined vaccine comprising FFA as one of a plurality of active components, such as a DPT combined vaccine, a DT combined vaccine, or a combined vaccine comprising FFA and at least one vaccine antigen selected from the group consisting of vaccine antigens other than FFA, such as influenza B vaccine antigen, inactivated poliomyelitis vaccine antigen, inactivated hepatitis B vaccine antigen, inactivated Japanese encephalitis vaccine antigen and the like, and a method for producing the above-mentioned vaccines in large quantities. Hereinbelow, explanation is made on the preparation of the FFA of the present invention, the preparation of a vaccine using the prepared FFA, the tests for evaluating the prepared vaccine, and the like.

(1) Seed microorganisms:

With respect to a microorganism used as a seed culture for obtaining the functional fragment antigen (FFA) of the present invention, there is no particular limitation as long as the microorganism has high toxin-producing ability. Examples of such microorganisms include the substrain of Clostridium tetani Harvard strain, and other C. tetani strains having substantially the same or higher producing ability for tetanus toxin, as or than that of the Harvard strain. Specifically, for example, it is preferred to use the Biken substrain having high toxin-producing ability (Reference Example 1), obtained by single colony isolation from the Harvard H47 strain, which is a known C. tetani strain derived from the Harvard strain [deposited with ATCC (American Type Culture Collection) under the accession No. 10779].

Further, as a seed microorganism, there can also be used a transformant microorganism obtained by a method in which a microorganism, such as yeast, Escherichia coli, Bacillus subtilis or the like, is transformed with a gene coding for FFA, using genetic engineering techniques. Specifically, for example, as a seed microorganism there can be used Escherichia coli transformed with a large-quantity expression vector having a DNA encoding FFA operably ligated thereto, which transformed E. coli is obtained in accordance with the method described in Reference Example 2 mentioned below.

(2) Medium:

Conventional media can be used for culturing the seed microorganism for obtaining FFA. For example, a conventional liquid medium for culturing an anaerobic microorganism can be used for culturing the above-mentioned seed microorganisms. Examples of such liquid media include cooked meat medium, PYG (Peptone, Yeast extract, Glucose) medium, GAM (Gifu Anaerobic Medium) broth, Veal infusion medium, thioglycolate medium, liver-liver broth, RCM (Reinforced Clostridial Medium) broth and DRCM (Differential Reinforced Clostridial Medium) broth. If desired, in order to improve the growth characteristics of the microorganisms and/or the maintenance of the low oxidation-reduction potential, a medium can be modified by replacement, addition or removal of components thereof, or by changing the amount of components thereof. Among these media, liver-liver broth is preferred for use in preparing a seed culture, and a modified Latham medium designed by the present inventor (Reference Example 3) is preferred for use in producing tetanus toxin from the seed culture.

(3) Culture conditions:

There is no particular limitation with respect to the conditions for culturing the seed microorganism for obtaining FFA. For example, a strain of C. tetani having high toxin-producing ability is cultured under culture conditions, in which the incubation temperature is from about 30 to about 37 °C, preferably from about 34 to about 36 °C, and the incubation time is from about 1 to about 8 days, particularly from about 1 to about 2 days for extracting the intracellular toxin, and

tetanus toxin into a nicked form. Therefore, for saving labor in the preparation process and achieving high yield, it is preferred to use, as a starting material, a culture supernatant or a culture filtrate each containing an extracellular tetanus toxin in a nicked form.

5 (5) Preliminary purification of tetanus toxin:

The whole tetanus toxin molecule in the starting material can be roughly purified by conventional methods. Examples of such conventional methods include salting out by using ammonium sulfate, alcohol precipitation, adsorption onto and desorption from a gel, and ultrafiltration by using commercially available membranes. In the present invention, 10 the whole tetanus toxin molecule obtained by these preliminary purification methods is referred to as "partially purified whole tetanus toxin".

(6) High purification of tetanus toxin:

15 The partially purified whole tetanus toxin, obtained by the method described in item (5) above, can be highly purified by, for example, a method using both density-gradient ultracentrifugation and equilibrium density-gradient ultracentrifugation (see Unexamined Japanese Patent Application Laid-Open Specification No. 07-89951), or a method using an appropriate combination of conventional methods, such as ultracentrifugation, gel filtration, ion-exchange chromatography and high performance liquid chromatography. In the present invention, a highly purified whole tetanus toxin 20 molecule obtained by these purification methods (hereinafter, frequently referred to simply as "highly purified tetanus toxin") can be used as a material for preparation of FFA of the present invention. The highly purified tetanus toxin needs to be confirmed with respect to its eligibility for use as whole tetanus toxin molecules. The confirmation of the eligibility can be performed by determining, for example, MLD (Minimum Lethal Dose; Reference Example 4), Lf unit (Unit of flocculation; Reference Example 5), protein content (Reference Example 6), or the like.

25 (7) Preparation of FFA:

In the present invention, FFA is prepared from the above-mentioned highly purified tetanus toxin. When the highly purified tetanus toxin used for preparation of FFA is prepared from intracellular tetanus toxin, it is first necessary to 30 digest mildly or split the intracellular tetanus toxin with a protease, such as trypsin or chymotrypsin, so as to convert the toxin into a nicked form. Two functionally complementary fragments of tetanus toxin can be prepared by a method comprising the steps of splitting a disulfide bridge present in the N-terminal of the purified tetanus toxin in the nicked form by a reducing agent, and splitting non-covalent bonds between groups on the tetanus toxin molecule by a denaturing agent. Examples of reducing agents include conventional reducing agents, such as sodium thioglycolate, dithiothreitol 35 (hereinafter referred to simply as "DTT"), glutathione, mercaptoethanol, hydrogen sulfide, sodium borohydride, sodium sulfide, ammonium sulfide and the like. As denaturing agents, conventional denaturing agents can be used. Examples of these agents include guanidine thiocyanate, guanidine hydrochloride, urea, sodium dodecyl sulfate and the like. In the present invention, DTT and urea are preferred. With respect to a preferred manner of the use of DTT, for example, the final concentration of DTT in a solution containing the toxin protein in an amount of from about 1 to about 10 mg/ml 40 is generally in the range of from 10 to 200 mM, preferably from 50 to 150 mM, and the reaction is conducted at 15 to 35 °C for 20 to 180 minutes. With respect to a preferred manner of the use of urea, for example, the final concentration of urea in a solution containing the toxin protein in an amount of from about 1 to about 10 mg/ml is generally in the range of from 0.5 to 10 M, preferably from 1 to 5 M, and the reaction is conducted at 5 to 35 °C for 10 seconds to 15 minutes. Each of these reagents is added to the starting material so that the concentration of the reagent falls within the above- 45 mentioned respective concentration range. In the present invention, DTT is used in the form of a solution (see Reference Example 8; hereinafter, this solution is referred to as "DTT Tris buffer"), and the solution is added to the starting material in an amount about 5 to about 50 times by volume the amount of the starting material, to react DTT with tetanus toxin. Urea is used in the form of a saturated solution or directly in the form of crystals.

As a result of the above-mentioned treatments with DTT and urea, the whole tetanus toxin molecule in the nicked

be prepared according to the method described below (Reference Example 13). The molecular weight, antigenic specificity, amino acid sequence and the like of each of the whole tetanus toxin molecule and FFA can be determined by, for example, SDS-PAGE (Reference Example 10), a precipitation reaction in gel (Reference Example 11) and a method using a peptide sequencer (Reference Example 12).

(8) Stabilization of FFA:

In the FFA of the present invention, a whole or most of the fragment A region, which is the active site exhibiting the toxicity, is absent, so that the FFA of the present invention has no toxicity. Therefore, the FFA as such can be used as a toxoid without detoxification. However, for stabilizing the stereochemical structure of FFA as an antigen, it is preferred to perform a stabilization (fixation) of the tetanus toxin FFA. Examples of fixatives (fixing or stabilizing reagent) include conventional detoxifying agents, such as formalin, glutaraldehyde, β -propiolactone and the like. For example, when formalin is used as a fixative, it is preferred that the volume ratio of formalin to the FFA solution is approximately within the range of from 0.0004 to 0.7 % (v/v). The fixation temperature is approximately within the range of from 3 to 37 °C and the fixation time is approximately within the range of from 5 to 180 days. When there is a danger that the antigenicity of FFA is deteriorated by the fixation, the fixation conditions are rendered moderate by lowering the concentration of the fixative, lowering the fixation temperature or adding neutral amino acids, such as glycine and serine, or basic amino acids, such as lysine and arginine. When free formaldehyde remains in the solution after the fixation, if desired, it can be neutralized by addition of sodium hydrogensulfite in an equivalent amount to the amount of the formaldehyde, or can be removed by filtration using a membrane or by dialysis. After the fixation treatment, the FFA solution is stored at 4 °C for subsequent use as a bulk solution of tetanus toxin vaccine for preparing a tetanus vaccine. After the fixation treatment, the treated whole tetanus toxin molecule and treated FFA are referred to as a "whole tetanus toxin toxoid" and an "FFA toxoid", respectively.

(9) Preparation of FFA tetanus vaccine:

The FFA toxoid bulk solution obtained in item (8) above can be diluted so as to obtain a vaccine comprising the FFA toxoid in an effective immunogenic amount. For example, the bulk solution can be diluted with an isotonic solution of salts, or with buffer or a medium for tissue culture so that the protein content of the vaccine becomes from 20 to 200 μ g for a single-antigen toxoid, or 8 to 80 μ g for an adsorbed toxoid.

When the bulk solution is diluted as mentioned above, a stabilizer for increasing the heat resistance of the vaccine or an adjuvant as a supplementary agent for increasing the immunopotency can be added to the vaccine solution. It is preferred that the stabilizer is added to the vaccine in an amount of from 0.01 to 10 % (w/v), and the adjuvant is added in an amount of from 0.1 to 50 mg per ml of the vaccine. Examples of stabilizers include saccharides, amino acids, gelatin hydrolyzate, human albumin and the like. Examples of adjuvants include gels capable of effecting sustained release of antigens, such as calcium phosphate, aluminum phosphate, aluminum sulfate, alumina, bentonite and the like; and antibody production inducing agents, such as muramylpeptide derivatives, Krestin, Picibanil and the like.

Subsequently, the single-antigen vaccine or adsorbed vaccine is dispensed into small containers, such as ampuls or vials. Then, the containers are sealed hermetically, for example, by fusion sealing, and the sealed vaccine is provided as a plain or adsorbed preparation. When the vaccine is lyophilized after dispensation, the vaccine can be provided as a lyophilized preparation. Further, the vaccine can be provided in the form of a combined vaccine preparation, such as a DT binary vaccine, a DPT ternary vaccine, a quaternary vaccine or the like.

Each of these preparations is subjected to various tests to verify the eligibility for use as a toxoid or a vaccine. That is, each of these preparations is subjected to various tests on effectiveness, safety and homogeneity in accordance with a relevant provision (such as a provision entitled "tetanus toxoid", a provision entitled "adsorbed tetanus toxoid", a provision entitled "diphtheria-tetanus combined toxoid", or a provision entitled "diphtheria-pertussis-tetanus combined vaccine") in the Notification No. 217 of the Japanese Ministry of Health and Welfare: "Seibutsugakuteki Seizai Kijun (Minimum Requirements for Biological Products), to thereby verify its eligibility for use as a vaccine. Only the verified preparations are put to practical use. With respect to the manner of administration, for example, a preparation is usually administered by subcutaneous or intramuscular injection in an amount of 0.5 ml per person. In the case of a lyophilized

"adsorbed tetanus toxoid" in the Notification No. 217 of the Japanese Ministry of Health and Welfare: "Seibutsugakuteki Seizai Kijun (Minimum Requirements for Biological Products)". In addition to the above-mentioned tests, in the present invention, the relative immunopotency of FFA to that of the whole tetanus toxoid is measured by performing a further potency assay using a challenge toxin, wherein the score method (Reference Example 15) is employed.

(11) Animal tests on the adverse side effects of FFA tetanus toxoid:

Animal tests on the side effects of the FFA tetanus vaccine of the present invention can be conducted in accordance with conventional methods. Examples of animal tests which can be employed for evaluating the adverse side effects of tetanus toxoid include the passive cutaneous anaphylaxis (PCA) test using rats which is based on an immediate type allergic reaction (Reference Example 17), the footpad reaction test using mice which is based on a delayed type allergic reaction (Reference Example 18), and the intradermal reaction test using guinea pigs (Reference Example 19) which test is based on a delayed type allergic reaction.

BEST MODE FOR CARRYING OUT THE INVENTION

Hereinbelow, the present invention will be described in more detail with reference to the following Examples and Reference Examples, but they should not be construed as limiting the scope of the present invention.

In the following Reference Examples, the guide-lines for carrying out the present invention are specifically shown.

[Reference Example 1] Isolation of a single colony of a C. tetani strain having high toxin-producing ability:

Colonies of C. tetani Harvard A47 strain are formed on a plate of Zeissler's blood agar (prepared by adding glucose and defibrinated bovine blood to a commercially available non-modified agar medium in such amounts as would give a final glucose concentration of 2 % (w/v) and a final defibrinated bovine blood concentration of 20 % (v/v), followed by mixing), and the formed colonies are individually inoculated into a modified Latham medium (see Reference Example 3 below) and incubated to obtain cultures. With respect to each of the obtained cultures, the LI value is measured in accordance with the method as described in Reference Example 5 below. From the above-mentioned colonies, the colony used for the culture having the highest LI value is used as a Biken substrain of the C. tetani Harvard A47 strain, which has high toxin-producing ability.

[Reference Example 2] Preparation of transformants having high toxin-producing ability for FFA:

A DNA of tetanus toxin gene [see EMBO JOURNAL, 5(10), 2495-2502, 1986 and Nucleic Acid Research 14(19), 7809-7812, 1986] is digested with the restriction enzymes, thereby obtaining a 2.7 kb DNA fragment (Stu I-Bsp HI) cod-

(continued)

Uracil	1.25 mg
Nicotinic acid	0.25 mg
Thiamine	0.25 mg
Riboflavin	0.25 mg
Pyridoxine	0.25 mg
Biotin	2.5 μ g
Vitamin B ₁₂	0.05 μ g
Folic acid	100 μ g
Iron(III) chloride (hexahydrate)	32 mg
Iron sulfate (heptahydrate)	0.2 g
(The pH was adjusted to 7.0 using 7N NaOH)	

[Reference Example 4] MLD (Minimum Lethal Dose):

20

0.1 to 0.5 ml of each of dilutions of the tetanus toxin-containing solution which have been prepared by successively diluting tetanus toxin at logarithmic intervals of $10^{0.5}$ is individually injected subcutaneously or intramuscularly to OF1 mice (weighing 20 to 25 g) at the of the thigh of the left hind leg. MLD is determined, based on the dose (log. of dose)-response (time to death) curve (see "Proceedings of the 6th International Conference on Tetanus (Lyon, 1981)" pp. 21-25 32).

[Reference Example 5] Lf unit (Unit of Flocculation):

The Lf value of a toxin solution can be measured by the Ramon's method. (Biken Journal, 7, 137-152, 1964). 1 Lf of the toxin, which is the amount of the toxin which reacts with 1 unit of the antitoxin, is measured. Also, the measurement of the above-mentioned Lf value can be conducted using SRID (Single Radial Immunodiffusion) (see Immunochimistry, 2, 235-254, 1965).

[Reference Example 6] Measurement of protein content:

35

The protein content is measured in accordance with the "modified method of Lowry et al." in which the color reaction of a protein and a phenol reagent is evaluated by colorimetry. Hereinafter, this method is simply referred to as a "phenol reagent method".

40 [Reference Example 7] Identification of protein fractions and comparison on protein concentrations between the protein

[Reference Example 10] Measurement of the molecular weights of proteins by using SDS-PAGE:

In the SDS-PAGE, an SDS-PAGE gel having a gel content of 7.5 % (w/v), 7.0 % (w/v) (containing 2M urea), 5.0 % (w/v) or the like can be used. As a buffer solution, for example, 10 mM Tris-77 mM glycine buffer (pH 8.6) can be used. After the electrophoresis, the gel is stained using Coomassie brilliant blue. The molecular weight of each of the proteins is individually determined from the ratio of the migration distance of the sample protein to the migration distance of the marker dye or the protein having known molecular weight. As a result of the measurements conducted in accordance with the above method, it is found that the molecular weights ($\times 10^4$) of the FFA and the whole tetanus toxin molecule are 100,000 and 150,000, respectively.

[Reference Example 11] Determination of the antigenic specificity by using double immunodiffusion:

The antigenic specificity is determined by the method of Ouchterlony using 1 % (w/v) agarose in 50 mM Tris-0.6 M Glycine buffer (containing 1 mM EDTA; pH 8.5) and horse anti-tetanus toxin serum from which nonspecific antibodies are removed. A cross-reaction of antigenicity is observed between the FFA and the whole tetanus toxin molecule.

[Reference Example 12] Determination of the amino acid sequence of the FFA:

The determination of the amino acid sequence of the FFA is conducted using an automatic amino acid sequencer, such as Applied Biosystem Procise Type 492 manufactured and sold by Perkin Elmer, U.S.A. The FFA obtained in Example 1 below has N-terminal amino acid sequence (7) of Fig. 2(a), and the FFA obtained in Example 7 below has N-terminal amino acid sequence (4) of Fig. 2(a). Further, by appropriately choosing the conditions for cultivation of *C. tetani*, an FFA having N-terminal amino acid sequence (8) of Fig. 2(a) can be obtained. FFA's respectively having N-terminal amino acid sequences (1) to (3) and (6) of Fig. 2(a) can be prepared from the intracellular tetanus toxin by appropriately choosing the conditions for digesting the intracellular tetanus toxin molecule with trypsin to convert the toxin molecule to a nicked form thereof, such as an enzyme concentration, a reaction time and a reaction temperature. An FFA preparation having N-terminal amino acid sequence (5) of Fig. 2(a) can be prepared by digesting the intracellular tetanus toxin molecule with chymotrypsin to convert the toxin molecule to a nicked form thereof. Thus, in the present invention, 8 different types of FFA's respectively having N-terminal amino acid sequences (1) to (8) of Fig. 2(a) can be obtained. These 8 types of FFA's may be used as an active component of a tetanus vaccine individually or in combination.

[Reference Example 13] Determination of the isoelectric point of FFA:

The isoelectric point of FFA is determined by an isoelectric focusing method using a commercially available gel, for example, a gel manufactured and sold under the tradename "Phast Gel IEP 3-9" by Pharmacia Biotech, Sweden. As a result of the measurements conducted in the Examples in accordance with the above method, it was found that the FFA of the present invention has an isoelectric point within the range of 7.25 ± 0.5 .

[Reference Example 14] Preparation of the whole tetanus toxin molecule:

The seed culture of the *C. tetani* strain obtained in Reference Example 1 is inoculated into a culture medium described in Reference Example 3 for 44 hours and the bacterial cells are harvested by centrifugation at $10,000 \times g$ at 4°C for 25 minutes. 1 M NaCl solution containing 0.1 M sodium citrate is added in an amount of 1/30 volume of the cell culture to extract the intracellular toxin by gentle agitation. The resultant mixture is stirred at 4°C overnight to extract

	Results	Score
5	The animal dies on the 1st day:	0
	The animal dies on the 2nd day:	1
	The animal dies on the 3rd day:	2
10	The animal dies or exhibits serious symptoms (such as a tonic convulsion, a difficulty in walking and a respiratory distress) between the 4th and 7th day:	3
	The animal survives through one week with slight symptoms [such as a local paralysis of the abdominal muscle on a side opposite to the side on which the injection had been made (this symptom is checked by observing the animal hung by its tail)]:	4
15	The animal survives through one week without any symptom:	8

20 The obtained scores are analyzed by a computer using a software designed for statistical analysis for the parallel-line assay, in which the correlation analysis of the scores is conducted in terms of uniformity, linearity and parallelity to evaluate the relative immunopotency of the tetanus toxoid to that of the standard tetanus whole toxin toxoid of a known international units.

[Reference Example 16] Intradermal reaction using Guinea pigs:

25 Three groups of guinea pigs are sensitized by intramuscular injection with 1 ml of tetanus vaccine (10 µg protein/ml) per 1 guinea pig, wherein the above-mentioned three groups of guinea pigs are sensitized with (1) commercially available partially purified whole toxin toxoid, (2) purified whole toxin toxoid and (3) FFA vaccine, respectively. After 4 weeks from the sensitization, the back of each of the guinea pigs is shaved and then, the guinea pigs are individually injected
30 intradermally with 0.1 ml of the above-mentioned tetanus toxoid onto the back thereof. The protein concentration of the tetanus toxin solution is varied (32.0, 10.0 and 3.2 µg) depending on the group to which a guinea pig belongs. 24 hours after the injection, with respect to each of the guinea pigs, the portion which has been injected is examined to see whether or not a skin reaction, an erythema, an induration and/or a necrosis occurs. When the occurrence of an erythema is observed, the diameter of the erythema is measured.

35 Example 1

(1) Production and purification of the whole tetanus toxin molecule (nicked form)

40 Into each of 20 tubes (diameter: 6 cm; height: 38 cm) containing 450 ml of a modified Latham medium (see Reference Example 3), 5 ml of the seed culture of a Biken substrain of the *C. tetani* Harvard A47 strain (see Reference Example 1) was inoculated. The tubes with loose cotton plugs were incubated at 35 °C for 5 days until the cultures became clear. These cultures were centrifuged at 10,000 x g at 4 °C for 30 minutes to obtain clean culture supernatants. 8.5
45 liters of the culture supernatants thus obtained as starting materials were filtered and used for purification of whole tetanus toxin molecules (nicked form).

A saturated solution (at 25 °C) of ammonium sulfate (pH 7.0) was added to and mixed with the starting material in an ice-water bath to salt out a fraction having an ammonium sulfate saturation of 20 % to 40 %. The obtained fraction was suspended in 65 ml of 0.06 M phosphate buffer (pH 7.5, 4 °C) (see Reference Example 9) to obtain a suspension having an ammonium sulfate saturation of 40 %. The obtained suspension was subjected to centrifugation at 15,000 x
50 g at 4 °C for 30 minutes to obtain a precipitate. The obtained precipitate was washed by resuspending the precipitate in 22 ml of the above-mentioned phosphate buffer, and the resultant was subjected to centrifugation under the same

was fractioned into 1 ml fractions. With respect to each of the fractions, an absorbance at 280 nm (see Reference Example 7) was measured. The fractions having a high absorbance were pooled, thereby obtaining a gel filtration eluate in a total volume of 5 ml.

With respect to the obtained eluate, the measurement of the protein content in accordance with the phenol reagent method (see Reference Example 6), the measurement of MLD per 1 mg of protein (see Reference Example 4) and the measurement of Lf per 1 mg of protein (see Reference Example 5) were conducted. As a result, the protein content of the eluate was 60 mg/ml, Lf was 400 and MLD was 3.5×10^7 .

Further, 0.2 ml of the eluate was subjected to high performance liquid chromatography (HPLC) using TSK G3000 SW column (inner diameter: 0.75 cm, length: 60 cm) (manufactured and sold by Tosoh Corp., Japan) equilibrated with 0.1 M phosphate buffer (pH 6.8), in which elution was carried out using the above-mentioned phosphate buffer (flow rate: 0.6 ml/minute) to thereby obtain fractions. The absorbance at 280 nm of each of the fractions was measured. As a result, a single sharp peak was observed, which indicates that the whole tetanus toxin molecule was highly purified. In the preparation of FFA described below, the eluate obtained above was used as a solution of a highly purified entire length tetanus toxin molecule (nicked form) (i.e., a highly purified tetanus toxin solution).

(2) Preparation of FFA

18 ml of a DTT-Tris buffer (see Reference Example 8) was added to 2 ml of the highly purified tetanus toxin solution, followed by mixing. The resultant mixture was reacted at 25 °C for 60 minutes to reduce the disulfide bridge present in the whole tetanus toxin molecule. The resultant mixture was treated with urea by adding 4.8 g of a solid urea to the mixture, followed by mixing to dissolve the solid urea in the mixture (final urea concentration: 4 M). To the resultant was added 20 ml of 50 mM Tris buffer (containing 0.6 M glycine, 1 mM EDTA and 1 mM DTT; pH 8.5) and the resultant mixture was condensed using Amicon Ultra-filtration System, thereby obtaining 3 ml of a condensate. The obtained condensate was subjected to gel filtration by using Ultrogel AcA 44 column (inner diameter: 1.5 cm, height: 90 cm) equilibrated with 50 mM Tris buffer (containing 0.6 M glycine, 1 mM EDTA and 1 mM DTT and 2 M urea; pH 8.5). In the gel filtration, elution was carried out using the above-mentioned 50 mM Tris buffer (flow rate: 5 ml/hr) and the resultant eluate was fractioned into 1.2 ml fractions. With respect to each of the fractions, the absorbance at 280 nm was measured. As a result, two peaks (peak 1 and peak 2) were observed. From the obtained fractions including two fractions each exhibiting a peak at 280 nm (i.e., a peak 1 fraction and a peak 2 fraction, wherein the peak 1 fraction was obtained earlier than the peak 2 fraction), a set of 5 fractions (total amount: 6 ml) successively obtained starting from the peak 1 fraction and another set of 5 fractions (total amount: 6 ml) successively obtained starting from the peak 2 fraction were collected to obtain a collected fraction 1 and a collected fraction 2, respectively.

Each of the highly purified tetanus toxin solution, the collected fraction 1 and the collected fraction 2 was subjected to SDS-PAGE (see Reference Example 8) for determination of the approximate molecular weight and to gel precipitation reaction (see Reference Example 9) for determination of the antigenicity. As a result, the molecular weights of the purified tetanus toxin, the collected fraction 1 and the collected fraction 2 were approximately 150,000, 100,000 and 50,000, respectively. With respect to the antigenicity, the cross-reaction of the antigenicity was observed between the highly purified tetanus toxin solution and each of the collected fraction 1 and the collected fraction 2, whereas no cross-reaction was observed between the collected fraction 1 fraction and the collected fraction 2, which indicates that these two types of collected fractions had completely different antigenicities. Further, the protein contents of the collected fraction 1 and the collected fraction 2 were determined by the phenol reagent method. The protein contents of the collected fraction 1 and the collected fraction 2 were 15 mg/ml and 8 mg/ml, respectively. From the above results, it was confirmed that the collected fraction 1 contains FFA. In the operations described below, the collected fraction 1 was used as an FFA solution.

(3) Stabilization of the FFA

4 ml of the FFA solution was dialyzed against 1/15 M phosphate buffer (pH 7.8) at 4 °C overnight. After completion of the dialysis, the above mentioned phosphate buffer was added to the dialysate with the final concentration of 1/15 M.

(4) Preparation of a sample FFA tetanus vaccine

The bulk FFA tetanus vaccine solution was diluted with a 0.85 % (w/v) NaCl solution so as to obtain a diluted solution having a final protein concentration of 50 µg/ml. An equivolume of $\text{Al}(\text{OH})_3$ gel suspension [$\text{Al}(\text{OH})_3$ content: 2 mg/ml] was added to the obtained diluted solution, followed by mixing. The resultant mixture was allowed to stand at 4 °C overnight so that the FFA was adsorbed on the $\text{Al}(\text{OH})_3$ gel, thereby obtaining a sample vaccine. With respect to the obtained sample vaccine, the activities thereof (immunopotency) and the safety thereof (toxicity or adverse side effects) were evaluated as described below. (5) Evaluation of the immunopotency of the sample FFA tetanus vaccine

The evaluation of the immunopotency of the sample FFA tetanus vaccine was conducted by experiments using mice. As a control, another vaccine was prepared in substantially the same manner as in the items (3) and (4) above, except that the solution of the whole tetanus toxin molecule prepared in Reference Example 14 was used. With respect to each of the sample vaccine and the control vaccine, serial 2.5-fold dilution with a 0.85 % (w/v) NaCl solution was conducted to obtain dilutions having different dilution ratios, which were administered to mice as described below, wherein at least three dilutions had dilution ratios such that the dilutions exhibited dose-response relationships falling within the linear region of the dose-response curve. Using the obtained vaccine dilutions (i.e., sample vaccine dilutions and control vaccine dilutions), the immunization of mice was conducted as follows. The sample vaccine dilutions (each having the amount of 0.5 ml) were, respectively, administered to 10 randomly selected ddy/s female mice (each weighing 22 to 26 g) by subcutaneous injection to the inside of the thigh of the left hind leg. Four weeks after the injection, each of the immunized mice was challenged with 100 LD₅₀ standard toxin (Lot TA-4B) (provided by the National Institute of Health of Japan) by subcutaneous injection. The above operations were also conducted using the control vaccine dilutions instead of the sample vaccine dilutions. After the above operations, observations were made over a week as to whether or not the mice were alive, and as to the symptoms of the mice which were alive. Results of the observations were evaluated by the score method (see Reference Example 15). The obtained scores were analyzed with respect to variance and correlation by a computer using a software for the statistical analysis. From the results of the statistical analysis, the relative immunopotency of the sample FFA tetanus vaccine (the ratio of the immunopotency of the sample vaccine relative to the immunopotency of the vaccine comprising the whole tetanus toxin toxoid, wherein the immunopotency of the control vaccine is defined as 1.0) was calculated. The above experiment was repeated four times, and the obtained values of the relative immunopotency were statistically analyzed by a computer. Results are shown in Table 1. The immunopotency of the sample FFA tetanus vaccine was substantially the same as that of the control vaccine comprising the whole tetanus toxin toxoid.

(5) Experiments using guinea pigs to evaluate the degree of adverse side reactions caused by the intradermal reaction of the sample FFA tetanus vaccine.

The intradermal reactions were conducted using guinea pigs (std. Hartley, weighing 300 to 350 g, 5 weeks old, female) (obtained from Japan SLC, Inc., Japan) in accordance with the method described in Reference Example 16. The sensitization of the guinea pigs was conducted as follows. As antigens for sensitizing the guinea pigs, use was made of the sample FFA tetanus vaccine (FFA), a commercially available tetanus toxoid presumably containing a whole tetanus toxin toxoid (conventional toxoid) and the vaccine comprising the purified whole tetanus toxin toxoid (whole toxin toxoid). Each of these antigens was diluted using a 0.85 % (w/v) NaCl solution so that the final protein concentration and the final $\text{Al}(\text{OH})_3$ concentration became 10 µg/ml and 0.2 mg/ml, respectively, thereby obtain the three types of antigen dilutions. The guinea pigs were randomly divided into nine groups each consisting of three guinea pigs, and the above-obtained three types of the antigen dilutions were, respectively, administered to three guinea pigs of each of the nine groups. After completion of the sensitization period (4 weeks), the sensitized guinea pigs were challenged by the above-mentioned antigens by the following method. With respect to each of the above-mentioned antigens, three types of dilutions thereof respectively having final protein concentrations of 3.2, 1.0 and 0.32 µg/ml were prepared using a 0.85 % (w/v) NaCl solution. The resultant nine types of dilutions (consisting of three types of dilutions of the FFA, three types of dilutions of the conventional toxoid and three types of dilutions of the whole toxin toxoid) were administered to

Example 2

(1) Preparation of the bulk FFA tetanus vaccine solution

5 0.5 Liter of a seed culture of a Biken substrain of *C. tetani* Harvard A47 strain was inoculated in 80 liters of the modified Latham medium contained in a stainless steel tank having a volume of 100 liters (diameter: 60 cm, height: 50 cm). The tank was sealed by means of a silicone sheet, and the seed culture was incubated at 35 °C for 6 days to thereby obtain a culture. The obtained culture was filtered through a celite-filter paper for sterilization, thereby obtaining 75 liters of a filtrate. The obtained filtrate was concentrated using the "pericon cassette system" (manufactured and sold by Mil-

10 lipore, U.S.A.), thereby obtaining 7 liters of a condensate. Using the obtained condensate as a starting material, the purification of the whole tetanus toxin molecule, the preparation of the FFA and the stabilization of the FFA were performed in substantially the same manner as in Example 1, thereby obtaining 450 ml of a bulk FFA tetanus vaccine solution having a protein concentration of 600 µg/ml.

15 (2) production of an FFA tetanus plain vaccine preparation

The bulk FFA tetanus vaccine solution obtained above was diluted using 1/75 M phosphate buffer (pH 6.5) so that the final protein concentration of the vaccine preparation would become 60 µg/ml. To the resultant dilution were individually added sucrose, L-arginine and Haemaccel (manufactured and sold by Hoechst Aktiengesellschaft, Germany) in

20 this order in amounts such that the final concentrations of sucrose, L-arginine and Haemaccel became 3 % (w/v), 1 % (w/v) and 2 % (w/v), respectively, followed by mixing to obtain a single-antigen vaccine preparation. The obtained preparation was dispensed in glass vials each having a volume of 1 ml, so that each vial contained 0.6 ml of the preparation, and then, the vials were sealed. The obtained single-antigen vaccine preparation was subjected to various tests in accordance with a provision entitled "tetanus toxoid" in the Notification No. 217 of the Japanese Ministry of Health and

25 Welfare: "Seibutsugakuteki Seizai Kijun (Minimum Requirements for Biological Products)". As a result, the obtained preparation was verified as a qualified vaccine.

Example 3

30 Production of an adsorbed FFA tetanus vaccine preparation

The bulk FFA tetanus vaccine solution obtained in Example 2 was diluted using 1/40 M phosphate buffer (pH 6.0) so that the final protein concentration of the vaccine preparation became 60 µg/ml. To the resultant dilution was added an aluminum phosphate gel in an amount such that the final aluminum phosphate gel concentration of the vaccine preparation became 0.2 ml/ml, thereby obtaining a mixture. The obtained mixture was stirred at 4 °C for 5 hours so as to

35 adsorb the FFA on the aluminum phosphate gel. The resultant mixture was subjected to centrifugation at 2000 rpm for 20 minutes at 4 °C to collect the gel. The collected gel was suspended in 1/75 M phosphate buffer (pH 6.5). To the resultant suspension were added sucrose, L-arginine and Haemaccel (manufactured and sold by Hoechst Aktiengesellschaft, Germany) in this order in amounts such that the final concentrations of sucrose, L-arginine and Haemaccel

40 became 3 % (w/v), 1 % (w/v) and 2 % (w/v), respectively, followed by mixing to obtain an adsorbed FFA tetanus vaccine preparation. The obtained preparation was dispensed in glass vials having a volume of 1 ml so that each vial contained 0.6 ml of the preparation, and then, the vials were sealed. The obtained adsorbed FFA tetanus vaccine preparation was subjected to various tests in accordance with a provision entitled "adsorbed tetanus toxoid" in the Notification No. 217 of the Japanese Ministry of Health and Welfare: "Seibutsugakuteki Seizai Kijun (Minimum Requirements for Biological

45 Products)". As a result, the obtained preparation was verified as a qualified vaccine.

combined vaccine" in the Notification No. 217 of the Japanese Ministry of Health and Welfare: "Seibutsugakuteki Seizai Kijun (Minimum Requirements for Biological Products)". As a result, the obtained preparation was verified as a qualified combined vaccine.

5 Example 5

Production of an adsorbed DT combined vaccine preparation using an FFA tetanus vaccine

An adsorbed FFA tetanus vaccine was prepared in substantially the same manner as in Example 3, except that the final protein concentration of the adsorbed FFA tetanus vaccine was changed to 120 µg/ml. An adsorbed diphtheria toxoid was prepared so that the toxoid concentration became two times that of the working concentration. The adsorbed FFA tetanus vaccine and the diphtheria toxoid were mixed together to obtain an adsorbed DPT combined vaccine preparation. The obtained preparation was dispensed in glass vials each having a volume of 1 ml so that each vial contained 0.6 ml of the preparation, and then, the vials were sealed. The obtained adsorbed DT combined vaccine preparation was subjected to various tests in accordance with a provision entitled "adsorbed diphtheria-tetanus combined vaccine" in the Notification No. 217 of the Japanese Ministry of Health and Welfare: "Seibutsugakuteki Seizai Kijun (Minimum Requirements for Biological Products)". As a result, the obtained preparation was verified as a qualified combined vaccine.

20 Example 6

Production of a dried FFA tetanus vaccine preparation

An FFA tetanus vaccine preparation was prepared in substantially the same manner as in Example 2. The obtained
25 FFA tetanus vaccine preparation was dispensed in glass vials each having a volume of 1 ml so that each vial contained
0.6 ml of the preparation, followed by freeze-drying to obtain a dried FFA tetanus vaccine preparation. Then, the vials
were sealed. One of the vials was unsealed and the dried FFA tetanus vaccine preparation was dissolved by sterilized
distilled water so as to obtain 0.6 ml of vaccine solution, and the obtained vaccine solution was subjected to various
tests in accordance with a provision entitled "tetanus toxoid" in the Notification No. 217 of the Japanese Ministry of
30 Health and Welfare "Seibutsugakuteki Seizai Kijun (Minimum Requirements for Biological Products)". As a result, the
obtained preparation was verified as a qualified combined vaccine.

Example 7

35 Production of a sample FFA tetanus vaccine and evaluation of the immunopotency thereof

The production of the vaccine using the extracellular toxin and the evaluation of the immunopotency of the produced vaccine were conducted in substantially the same manner as in Example 1, except that the conditions employed were changed as follows.

The time for culturing the seed culture of *C. letani* at 35 °C was changed to 6 days. The preparation of the FFA from the solution of the whole tetanus toxin molecule (nicked form) obtained by gel filtration using Ultrogel AcA 34 column was conducted as follows. A DTT-Tris buffer (see Reference Example 8) was added to the above-mentioned solution of the whole tetanus toxin molecule in an amount of 1 ml per 2 mg of the solution of the whole tetanus toxin molecule, followed by mixing. Then, the reaction was performed at 25 °C for 60 minutes to reduce the disulfide bridge present in the toxin molecule. Subsequently, the resultant reaction mixture was treated with urea by addition of a solid urea in an amount such that the final urea concentration became 4 M. The reaction mixture was applied to PD10 column (manufactured and sold by Pharmacia Biotech, Sweden) equilibrated with the Buffer A (0.2 M Tris-HCl, pH 7.5, 0.1 M NaCl).

using mice in substantially the same manner as in item (6) of Example 1. Results are shown in Table 3. As can be clearly seen from Table 3, the immunopotency of the sample FFA tetanus vaccine had substantially the same level as that of the control vaccine comprising the whole tetanus toxin toxoid (i. e., a conventional tetanus toxoid).

Table 1

Sample toxoid	Relative immunopotency	Fiducial limit (p = 0.95)
Purified whole toxin toxoid	1.0	
FFA	0.970	0.636-1.478
Purified whole toxin toxoid : Vaccine comprising the whole tetanus toxin toxoid FFA : Tetanus vaccine comprising the FFA		

Table 2

Amount of antigen administered by intradermal injection (μg)		Antigen used for sensitization and the degree of intradermal reaction		
		Conventional toxoid	Purified whole tetanus toxoid	FFA
Conventional toxoid	3.2	+6	+6	+3
	1.0	+6	+5	+2
	0.32	+5	+5	+1
Purified whole tetanus toxoid	3.2	+6	+6	+1
	1.0	+4	+5	+1
	0.32	+4	+4	*0
FFA	3.2	+5	+5	+1
	1.0	+3	+3	*0
	0.32	+1	+2	*0
Control	0	*0	*0	*0

Conventional toxoid : Commercially available tetanus toxoid

Purified whole tetanus toxoid : Vaccine comprising a whole tetanus toxin toxoid

FFA : Tetanus vaccine comprising the FFA

The degree of intradermal reaction is shown, using an indication selected from seven indications (*0) to (+6), in accordance with the following criteria, based on the size (E) of the erythema wherein E is a value of the formula:

major diameter (mm) of the erythema \times minor diameter (mm) of the erythema :

*0 : ($0 \leq E < 1$), +1 : ($1 \leq E < 20$), +2 : ($20 \leq E < 40$),

+3 : ($40 \leq E < 60$), +4 : ($60 \leq E < 80$),

+5 : ($80 \leq E < 100$), and +6 : ($100 \leq E$).

Table 3

Sample toxoid	Relative immunopotency	Fiducial limits ($p = 0.95$)
Purified whole toxin toxoid	1.0	
FFA	1.158	0.563-2.382
Purified whole toxin toxoid : Vaccine comprising the whole tetanus toxin toxoid FFA : Tetanus vaccine comprising the FFA		

SEQUENCE LISTING

5 SEQ ID NO. : 1

SEQUENCE LENGTH : 1315

SEQUENCE TYPE : amino acid

10 TOPOLOGY : linear

MOLECULE TYPE : peptide

15 SEQUENCE DESCRIPTION

Met Pro Ile Thr Ile Asn Asn Phe Arg Tyr Ser Asp Pro Val Asn

1 5 10 15

Asn Asp Thr Ile Ile Met Met Glu Pro Pro Tyr Cys Lys Gly Leu

20 25 30

25 Asp Ile Tyr Tyr Lys Ala Phe Lys Ile Thr Asp Arg Ile Trp Ile

35 40 45

30 Val Pro Glu Arg Tyr Glu Phe Gly Thr Lys Pro Glu Asp Phe Asn

50 55 60

Pro Pro Ser Ser Leu Ile Glu Gly Ala Ser Glu Tyr Tyr Asp Pro

35 65 70 75

Asn Tyr Leu Arg Thr Asp Ser Asp Lys Asp Arg Phe Leu Gln Thr

40 80 85 90

Met Val Lys Leu Phe Asn Arg Ile Lys Asn Asn Val Ala Gly Glu

95 100 105

45 Ala Leu Leu Asp Lys Ile Ile Asn Ala Ile Pro Tyr Leu Gly Asn

110 115 120

50 Ser Tyr Ser Leu Leu Asp Lys Phe Asp Thr Asn Ser Asn Ser Val

125 130 135

55

	Ser Phe Asn Leu Leu Glu Gln Asp Pro Ser Gly Ala Thr Thr Lys		
5	140	145	150
	Ser Ala Met Leu Thr Asn Leu Ile Ile Phe Gly Pro Gly Pro Val		
	155	160	165
10	Leu Asn Lys Asn Glu Val Arg Gly Ile Val Leu Arg Val Asp Asn		
	170	175	180
15	Lys Asn Tyr Phe Pro Cys Arg Asp Gly Phe Gly Ser Ile Met Gln		
	185	190	195
	Met Ala Phe Cys Pro Glu Tyr Val Pro Thr Phe Asp Asn Val Ile		
20	200	205	210
	Glu Asn Ile Thr Ser Leu Thr Ile Gly Lys Ser Lys Tyr Phe Gln		
	215	220	225
25	Asp Pro Ala Leu Leu Leu Met His Glu Leu Ile His Val Leu His		
	230	235	240
30	Gly Leu Tyr Gly Met Gln Val Ser Ser His Glu Ile Ile Pro Ser		
	245	250	255
	Lys Gln Glu Ile Tyr Met Gln His Thr Tyr Pro Ile Ser Ala Glu		
35	260	265	270
	Glu Leu Phe Thr Phe Gly Gly Gln Asp Ala Asn Leu Ile Ser Ile		
	275	280	285
40	Asp Ile Lys Asn Asp Leu Tyr Glu Lys Thr Leu Asn Asp Tyr Lys		
	290	295	300
45	Ala Ile Ala Asn Lys Leu Ser Gln Val Thr Ser Cys Asn Asp Pro		
	305	310	315
	Asn Ile Asp Ile Asp Ser Tyr Lys Gln Ile Tyr Gln Gln Lys Tyr		
50	320	325	330

55

	Gln Phe Asp Lys Asp Ser Asn Gly Gln Tyr Ile Val Asn Glu Asp		
5	335	340	345
	Lys Phe Gln Ile Leu Tyr Asn Ser Ile Met Tyr Gly Phe Thr Glu		
	350	355	360
10	Ile Glu Leu Gly Lys Lys Phe Asn Ile Lys Thr Arg Leu Ser Tyr		
	365	370	375
15	Phe Ser Met Asn His Asp Pro Val Lys Ile Pro Asn Leu Leu Asp		
	380	385	390
	Asp Thr Ile Tyr Asn Asp Thr Glu Gly Phe Asn Ile Glu Ser Lys		
20	395	400	405
	Asp Leu Lys Ser Glu Tyr Lys Gly Gln Asn Met Arg Val Asn Thr		
25	410	415	420
	Asn Ala Phe Arg Asn Val Asp Gly Ser Gly Leu Val Ser Lys Leu		
	425	430	435
30	Ile Gly Leu Cys Lys Lys Ile Ile Pro Pro Thr Asn Ile Arg Glu		
	440	445	450
35	Asn Leu Tyr Asn Arg Thr Ala Ser Leu Thr Asp Leu Gly Gly Glu		
	455	460	465
	Leu Cys Ile Lys Ile Lys Asn Glu Asp Leu Thr Phe Ile Ala Glu		
40	470	475	480
	Lys Asn Ser Phe Ser Glu Glu Pro Phe Gln Asp Glu Ile Val Ser		
45	485	490	495
	Tyr Asn Thr Lys Asn Lys Pro Leu Asn Phe Asn Tyr Ser Leu Asp		
	500	505	510
50	Lys Ile Ile Val Asp Tyr Asn Leu Gln Ser Lys Ile Thr Leu Pro		
	515	520	525

55

	Asn Asp Arg Thr Thr Pro Val Thr Lys Gly Ile Pro Tyr Ala Pro	
5	530	535 540
	Glu Tyr Lys Ser Asn Ala Ala Ser Thr Ile Glu Ile His Asn Ile	
	545	550 555
10	Asp Asp Asn Thr Ile Tyr Gln Tyr Leu Tyr Ala Gln Lys Ser Pro	
	560	565 570
15	Thr Thr Leu Gln Arg Ile Thr Met Thr Asn Ser Val Asp Asp Ala	
	575	580 585
	Leu Ile Asn Ser Thr Lys Ile Tyr Ser Tyr Phe Pro Ser Val Ile	
20	590	595 600
	Ser Lys Val Asn Gln Gly Ala Gln Gly Ile Leu Phe Leu Gln Trp	
	605	610 615
25	Val Arg Asp Ile Ile Asp Asp Phe Thr Asn Glu Ser Ser Gln Lys	
	620	625 630
30	Thr Thr Ile Asp Lys Ile Ser Asp Val Ser Thr Ile Val Pro Tyr	
	635	640 645
	Ile Gly Pro Ala Leu Asn Ile Val Lys Gln Gly Tyr Glu Gly Asn	
35	650	655 660
	Phe Ile Gly Ala Leu Glu Thr Thr Gly Val Val Leu Leu Leu Glu	
40	665	670 675
	Tyr Ile Pro Glu Ile Thr Leu Pro Val Ile Ala Ala Leu Ser Ile	
	680	685 690
45	Ala Glu Ser Ser Thr Gln Lys Glu Lys Ile Ile Lys Thr Ile Asp	
	695	700 705
50	Asn Phe Leu Glu Lys Arg Tyr Glu Lys Trp Ile Glu Val Tyr Lys	
	710	715 720

55

	Leu Val Lys Ala Lys Trp Leu Gly Thr Val Asn Thr Gln Phe Gln		
	725	730	735
5	Lys Arg Ser Tyr Gln Met Tyr Arg Ser Leu Glu Tyr Gln Val Asp		
	740	745	750
10	Ala Ile Lys Lys Ile Ile Asp Tyr Glu Tyr Lys Ile Tyr Ser Gly		
	755	760	765
15	Pro Asp Lys Glu Gln Ile Ala Asp Glu Ile Asn Asn Leu Lys Asn		
	770	775	780
	Lys Leu Glu Glu Lys Ala Asn Lys Ala Met Ile Asn Ile Asn Ile		
20	785	790	795
	Phe Met Arg Glu Ser Ser Arg Ser Phe Leu Val Asn Gln Met Ile		
	800	805	810
25	Asn Glu Ala Lys Lys Gln Leu Leu Glu Phe Asp Thr Gln Ser Lys		
	815	820	825
30	Asn Ile Leu Met Gln Tyr Ile Lys Ala Asn Ser Lys Phe Ile Gly		
	830	835	840
	Ile Thr Glu Leu Lys Lys Leu Glu Ser Lys Ile Asn Lys Val Phe		
35	845	850	855
	Ser Thr Pro Ile Pro Phe Ser Tyr Ser Lys Asn Leu Asp Cys Trp		
40	860	865	870
	Val Asp Asn Glu Glu Asp Ile Asp Val Ile Leu Lys Lys Ser Thr		
	875	880	885
45	Ile Leu Asn Leu Asp Ile Asn Asn Asp Ile Ile Ser Asp Ile Ser		
	890	895	900
50	Gly Phe Asn Ser Ser Val Ile Thr Tyr Pro Asp Ala Gln Leu Val		
	905	910	915

55

	Pro Gly Ile Asn Gly Lys Ala Ile His Leu Val Asn Asn Glu Ser		
5	920	925	930
	Ser Glu Val Ile Val His Lys Ala Met Asp Ile Glu Tyr Asn Asp		
	935	940	945
10	Met Phe Asn Asn Phe Thr Val Ser Phe Trp Leu Arg Val Pro Lys		
	950	955	960
15	Val Ser Ala Ser His Leu Glu Gln Tyr Gly Thr Asn Glu Tyr Ser		
	965	970	975
	Ile Ile Ser Ser Met Lys Lys His Ser Leu Ser Ile Gly Ser Gly		
20	980	985	990
	Trp Ser Val Ser Leu Lys Gly Asn Asn Leu Ile Trp Thr Leu Lys		
	995	1000	1005
25	Asp Ser Ala Gly Glu Val Arg Gln Ile Thr Phe Arg Asp Leu Pro		
	1010	1015	1020
30	Asp Lys Phe Asn Ala Tyr Leu Ala Asn Lys Trp Val Phe Ile Thr		
	1025	1030	1035
	Ile Thr Asn Asp Arg Leu Ser Ser Ala Asn Leu Tyr Ile Asn Gly		
35	1040	1045	1050
	Val Leu Met Gly Ser Ala Glu Ile Thr Gly Leu Gly Ala Ile Arg		
40	1055	1060	1065
	Glu Asp Asn Asn Ile Thr Leu Lys Leu Asp Arg Cys Asn Asn Asn		
	1070	1075	1080
45	Asn Gln Tyr Val Ser Ile Asp Lys Phe Arg Ile Phe Cys Lys Ala		
	1085	1090	1095
50	Leu Asn Pro Lys Glu Ile Glu Lys Leu Tyr Thr Ser Tyr Leu Ser		
	1100	1105	1110

55

	Ile Thr Phe Leu Arg Asp Phe Trp Gly Asn Pro Leu Arg Tyr Asp		
5	1115	1120	1125
	Thr Glu Tyr Tyr Leu Ile Pro Val Ala Ser Ser Ser Lys Asp Val		
	1130	1135	1140
10	Gln Leu Lys Asn Ile Thr Asp Tyr Met Tyr Leu Thr Asn Ala Pro		
	1145	1150	1155
15	Ser Tyr Thr Asn Gly Lys Leu Asn Ile Tyr Tyr Arg Arg Leu Tyr		
	1160	1165	1170
	Asn Gly Leu Lys Phe Ile Ile Lys Arg Tyr Thr Pro Asn Asn Glu		
20	1175	1180	1185
	Ile Asp Ser Phe Val Lys Ser Gly Asp Phe Ile Lys Leu Tyr Val		
25	1190	1195	1200
	Ser Tyr Asn Asn Asn Glu His Ile Val Gly Tyr Pro Lys Asp Gly		
	1205	1210	1215
30	Asn Ala Phe Asn Asn Leu Asp Arg Ile Leu Arg Val Gly Tyr Asn		
	1220	1225	1230
35	Ala Pro Gly Ile Pro Leu Tyr Lys Lys Met Glu Ala Val Lys Leu		
	1235	1240	1245
	Arg Asp Leu Lys Thr Tyr Ser Val Gln Leu Lys Leu Tyr Asp Asp		
40	1250	1255	1260
	Lys Asn Ala Ser Leu Gly Leu Val Gly Thr His Asn Gly Gln Ile		
45	1265	1270	1275
	Gly Asn Asp Pro Asn Arg Asp Ile Leu Ile Ala Ser Asn Trp Tyr		
	1280	1285	1290
50	Phe Asn His Leu Lys Asp Lys Ile Leu Gly Cys Asp Trp Tyr Phe		
	1295	1300	1305

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Val Pro Thr Asp Glu Gly Trp Thr Asn Asp

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10 INDUSTRIAL APPLICABILITY

15 According to the present invention, an FFA (tetanus toxin functional fragment antigen) for a tetanus vaccine is provided, which is advantageous not only in that it is extremely excellent with respect to the diminution of adverse side effects, as compared to the conventional tetanus toxoid, but also in that it has an immunopotency which is substantially the same as that of a conventional tetanus toxoid.

By the use of the FFA of the present invention as an active component for a tetanus vaccine, there can be provided a tetanus vaccine which is not only extremely excellent with respect to the diminution of adverse side effects, as compared to a conventional tetanus toxoid vaccine, but also has an immunopotency which is substantially the same as that of a conventional tetanus toxoid vaccine.

27 Further, the above-mentioned tetanus vaccine can also be provided in the form of a combined vaccine comprising the tetanus vaccine and at least one vaccine other than the tetanus vaccine, such as a pertussis vaccine and a diphtheria vaccine.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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(F) POSTAL CODE: 565

(ii) TITLE OF INVENTION: TETANUS TOXIN FUNCTIONAL FRAGMENT
ANTIGEN AND TETANUS VACCINE

(iii) NUMBER OF SEQUENCES: 9

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(F) POSTAL CODE: 768

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0. Version #1.30

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: 97907448.1 (PCT/JP97/00976)

(B) FILING DATE: 24-MAR-1997

(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: JP 8-106053

(B) FILING DATE: 23-MAR-1996

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Blake, John Henry Francis

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(C) REFERENCE/DOCKET NUMBER: JHB/97-1011/JLH

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 0875-25-4171
 (B) TELEFAX: 0875-25-4171

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1315
 (B) TYPE: amino acid
 (C) STRANDEDNESS: unknown
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Clostridium tetani

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Met	Pro	Ile	Thr	Ile	Asn	Asn	Phe	Arg	Tyr	Ser	Asp	Pro	Val	Asn	Asn	1	5	10	15
Asp	Thr	Ile	Ile	Met	Met	Glu	Pro	Pro	Tyr	Cys	Lys	Gly	Leu	Asp	Ile	20	25	30	
Tyr	Tyr	Lys	Ala	Phe	Lys	Ile	Thr	Asp	Arg	Ile	Trp	Ile	Val	Pro	Glu	35	40	45	
Arg	Tyr	Glu	Phe	Gly	Thr	Lys	Pro	Glu	Asp	Phe	Asn	Pro	Pro	Ser	Ser	50	55	60	
Leu	Ile	Glu	Gly	Ala	Ser	Glu	Tyr	Tyr	Asp	Pro	Asn	Tyr	Leu	Arg	Thr	65	70	75	80
Asp	Ser	Asp	Lys	Asp	Arg	Phe	Leu	Gln	Thr	Met	Val	Lys	Leu	Phe	Asn	85	90	95	
Arg	Ile	Lys	Asn	Asn	Val	Ala	Gly	Glu	Ala	Leu	Leu	Asp	Lys	Ile	Ile	100	105	110	
Asn	Ala	Ile	Pro	Tyr	Leu	Gly	Asn	Ser	Tyr	Ser	Leu	Leu	Asp	Lys	Phe	115	120	125	
Asp	Thr	Asn	Ser	Asn	Ser	Val	Ser	Phe	Asn	Leu	Leu	Glu	Gln	Asp	Pro	130	135	140	
Ser	Gly	Ala	Thr	Thr	Lys	Ser	Ala	Met	Leu	Thr	Asn	Leu	Ile	Ile	Phe	145	150	155	160

Gly Pro Gly Pro Val Leu Asn Lys Asn Glu Val Arg Gly Ile Val Leu
 165 170 175
 5 Arg Val Asp Asn Lys Asn Tyr Phe Pro Cys Arg Asp Gly Phe Gly Ser
 180 185 190
 10 Ile Met Gln Met Ala Phe Cys Pro Glu Tyr Val Pro Thr Phe Asp Asn
 195 200 205
 Val Ile Glu Asn Ile Thr Ser Leu Thr Ile Gly Lys Ser Lys Tyr Phe
 210 215 220
 15 Gln Asp Pro Ala Leu Leu Leu Met His Glu Leu Ile His Val Leu His
 225 230 235 240
 Gly Leu Tyr Gly Met Gln Val Ser Ser His Glu Ile Ile Pro Ser Lys
 245 250 255
 20 Gln Glu Ile Tyr Met Gln His Thr Tyr Pro Ile Ser Ala Glu Glu Leu
 260 265 270
 25 Phe Thr Phe Gly Gly Gln Asp Ala Asn Leu Ile Ser Ile Asp Ile Lys
 275 280 285
 Asn Asp Leu Tyr Glu Lys Thr Leu Asn Asp Tyr Lys Ala Ile Ala Asn
 290 295 300
 30 Lys Leu Ser Gln Val Thr Ser Cys Asn Asp Pro Asn Ile Asp Ile Asp
 305 310 315 320
 Ser Tyr Lys Gln Ile Tyr Gln Gln Lys Tyr Gln Phe Asp Lys Asp Ser
 325 330 335
 35 Asn Gly Gln Tyr Ile Val Asn Glu Asp Lys Phe Gln Ile Leu Tyr Asn
 340 345 350
 40 Ser Ile Met Tyr Gly Phe Thr Glu Ile Glu Leu Gly Lys Lys Phe Asn
 355 360 365
 Ile Lys Thr Arg Leu Ser Tyr Phe Ser Met Asn His Asp Pro Val Lys
 370 375 380
 45 Ile Pro Asn Leu Leu Asp Asp Thr Ile Tyr Asn Asp Thr Glu Gly Phe
 385 390 395 400
 Asn Ile Glu Ser Lys Asp Leu Lys Ser Glu Tyr Lys Gly Gln Asn Met
 405 410 415
 50 Arg Val Asn Thr Asn Ala Phe Arg Asn Val Asp Gly Ser Gly Leu Val
 55

	420	425	430
5	Ser Lys Leu Ile Gly Leu Cys Lys Lys Ile Ile Pro Pro Thr Asn Ile 435 440 445		
	Arg Glu Asn Leu Tyr Asn Arg Thr Ala Ser Leu Thr Asp Leu Gly Gly 450 455 460		
10	Glu Leu Cys Ile Lys Ile Lys Asn Glu Asp Leu Thr Phe Ile Ala Glu 465 470 475 480		
15	Lys Asn Ser Phe Ser Glu Glu Pro Phe Gln Asp Glu Ile Val Ser Tyr 485 490 495		
	Asn Thr Lys Asn Lys Pro Leu Asn Phe Asn Tyr Ser Leu Asp Lys Ile 500 505 510		
20	Ile Val Asp Tyr Asn Leu Gln Ser Lys Ile Thr Leu Pro Asn Asp Arg 515 520 525		
	Thr Thr Pro Val Thr Lys Gly Ile Pro Tyr Ala Pro Glu Tyr Lys Ser 530 535 540		
25	Asn Ala Ala Ser Thr Ile Glu Ile His Asn Ile Asp Asp Asn Thr Ile 545 550 555 560		
30	Tyr Gln Tyr Leu Tyr Ala Gln Lys Ser Pro Thr Thr Leu Gln Arg Ile 565 570 575		
	Thr Met Thr Asn Ser Val Asp Asp Ala Leu Ile Asn Ser Thr Lys Ile 580 585 590		
35	Tyr Ser Tyr Phe Pro Ser Val Ile Ser Lys Val Asn Gln Gly Ala Gln 595 600 605		
40	Gly Ile Leu Phe Leu Gln Trp Val Arg Asp Ile Ile Asp Asp Phe Thr 610 615 620		
	Asn Glu Ser Ser Gln Lys Thr Thr Ile Asp Lys Ile Ser Asp Val Ser 625 630 635 640		
45	Thr Ile Val Pro Tyr Ile Gly Pro Ala Leu Asn Ile Val Lys Gln Gly 645 650 655		
	Tyr Glu Gly Asn Phe Ile Gly Ala Leu Glu Thr Thr Gly Val Val Leu 660 665 670		
50	Leu Leu Glu Tyr Ile Pro Glu Ile Thr Leu Pro Val Ile Ala Ala Leu 675 680 685		

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Ser Ile Ala Glu Ser Ser Thr Gln Lys Glu Lys Ile Ile Lys Thr Ile
 690 695 700
 5 Asp Asn Phe Leu Glu Lys Arg Tyr Glu Lys Trp Ile Glu Val Tyr Lys
 705 710 715 720
 Leu Val Lys Ala Lys Trp Leu Gly Thr Val Asn Thr Gln Phe Gln Lys
 725 730 735
 10 Arg Ser Tyr Gln Met Tyr Arg Ser Leu Glu Tyr Gln Val Asp Ala Ile
 740 745 750
 Lys Lys Ile Ile Asp Tyr Glu Tyr Lys Ile Tyr Ser Gly Pro Asp Lys
 755 760 765
 Glu Gln Ile Ala Asp Glu Ile Asn Asn Leu Lys Asn Lys Leu Glu Glu
 770 775 780
 20 Lys Ala Asn Lys Ala Met Ile Asn Ile Asn Ile Phe Met Arg Glu Ser
 785 790 795 800
 Ser Arg Ser Phe Leu Val Asn Gln Met Ile Asn Glu Ala Lys Lys Gln
 805 810 815
 25 Leu Leu Glu Phe Asp Thr Gln Ser Lys Asn Ile Leu Met Gln Tyr Ile
 820 825 830
 Lys Ala Asn Ser Lys Phe Ile Gly Ile Thr Glu Leu Lys Lys Leu Glu
 835 840 845
 Ser Lys Ile Asn Lys Val Phe Ser Thr Pro Ile Pro Phe Ser Tyr Ser
 850 855 860
 35 Lys Asn Leu Asp Cys Trp Val Asp Asn Glu Glu Asp Ile Asp Val Ile
 865 870 875 880
 Leu Lys Lys Ser Thr Ile Leu Asn Leu Asp Ile Asn Asn Asp Ile Ile
 885 890 895
 40 Ser Asp Ile Ser Gly Phe Asn Ser Ser Val Ile Thr Tyr Pro Asp Ala
 900 905 910
 Gln Leu Val Pro Gly Ile Asn Gly Lys Ala Ile His Leu Val Asn Asn
 915 920 925
 Glu Ser Ser Glu Val Ile Val His Lys Ala Met Asp Ile Glu Tyr Asn
 930 935 940
 50 Asp Met Phe Asn Asn Phe Thr Val Ser Phe Trp Leu Arg Val Pro Lys
 945 950 955 960
 55

Val Ser Ala Ser His Leu Glu Gln Tyr Gly Thr Asn Glu Tyr Ser Ile
 965 970 975
 5 Ile Ser Ser Met Lys Lys His Ser Leu Ser Ile Gly Ser Gly Trp Ser
 980 985 990
 Val Ser Leu Lys Gly Asn Asn Leu Ile Trp Thr Leu Lys Asp Ser Ala
 995 1000 1005
 10 Gly Glu Val Arg Gln Ile Thr Phe Arg Asp Leu Pro Asp Lys Phe Asn
 1010 1015 1020
 Ala Tyr Leu Ala Asn Lys Trp Val Phe Ile Thr Ile Thr Asn Asp Arg
 1025 1030 1035 1040
 15 Leu Ser Ser Ala Asn Leu Tyr Ile Asn Gly Val Leu Met Gly Ser Ala
 1045 1050 1055
 20 Glu Ile Thr Gly Leu Gly Ala Ile Arg Glu Asp Asn Asn Ile Thr Leu
 1060 1065 1070
 Lys Leu Asp Arg Cys Asn Asn Asn Asn Gln Tyr Val Ser Ile Asp Lys
 1075 1080 1085
 25 Phe Arg Ile Phe Cys Lys Ala Leu Asn Pro Lys Glu Ile Glu Lys Leu
 1090 1095 1100
 Tyr Thr Ser Tyr Leu Ser Ile Thr Phe Leu Arg Asp Phe Trp Gly Asn
 1105 1110 1115 1120
 30 Pro Leu Arg Tyr Asp Thr Glu Tyr Tyr Leu Ile Pro Val Ala Ser Ser
 1125 1130 1135
 35 Ser Lys Asp Val Gln Leu Lys Asn Ile Thr Asp Tyr Met Tyr Leu Thr
 1140 1145 1150
 Asn Ala Pro Ser Tyr Thr Asn Gly Lys Leu Asn Ile Tyr Tyr Arg Arg
 1155 1160 1165
 40 Leu Tyr Asn Gly Leu Lys Phe Ile Ile Lys Arg Tyr Thr Pro Asn Asn
 1170 1175 1180
 Glu Ile Asp Ser Phe Val Lys Ser Gly Asp Phe Ile Lys Leu Tyr Val
 1185 1190 1195 1200
 45 Ser Tyr Asn Asn Asn Glu His Ile Val Gly Tyr Pro Lys Asp Gly Asn
 1205 1210 1215
 50 Ala Phe Asn Asn Leu Asp Arg Ile Leu Arg Val Gly Tyr Asn Ala Pro
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(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: N-terminal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Clostridium tetani

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Ile Ile Pro Pro Thr Asn Asn Ile Arg Glu Asn Leu Tyr Asn Arg Thr
1 5 10 15

Ala Ser Leu Thr Asp Leu Gly Gly Glu Leu Cys Ile Lys
20 25

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20
(B) TYPE: amino acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: N-terminal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Clostridium tetani

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Glu Asn Leu Tyr Asn Arg Thr Ala Ser Leu Thr Asp Leu Gly Gly Glu
1 5 10 15

Leu Cys Ile Lys
20

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 19
(B) TYPE: amino acid

(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

5

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: N-terminal

10

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Clostridium tetani

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Asn Leu Tyr Asn Arg Thr Ala Ser Leu Thr Asp Leu Gly Gly Glu Leu
1 5 10 15

20

Cys Ile Lys

(2) INFORMATION FOR SEQ ID NO:6:

25

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 16

(B) TYPE: amino acid

(C) STRANDEDNESS: unknown

(D) TOPOLOGY: unknown

30

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: N-terminal

35

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Clostridium tetani

40

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Asn Arg Thr Ala Ser Leu Thr Asp Leu Gly Gly Glu Leu Cys Ile Lys
1 5 10 15

45

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 14

50

(B) TYPE: amino acid

(C) STRANDEDNESS: unknown

(D) TOPOLOGY: unknown

55

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: N-terminal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Clostridium tetani

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Thr Ala Ser Leu Thr Asp Leu Gly Gly Glu Leu Cys Ile Lys
1 5 10

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 12

(B) TYPE: amino acid

(C) STRANDEDNESS: unknown

(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: N-terminal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Clostridium tetani

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Ser Leu Thr Asp Leu Gly Gly Glu Leu Cys Ile Lys
1 5 10

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 7

(B) TYPE: amino acid

(C) STRANDEDNESS: unknown

(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: N-terminal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Clostridium tetani

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Gly Gly Glu Leu Cys Ile Lys
 1 5

Claims

1. A tetanus toxin functional fragment antigen, comprising at least one fragment which is substantially the same as that obtained by a process comprising the steps of splitting at least one peptide bond selected from peptide bonds individually connecting mutually adjacent amino acid residues in a partial amino acid sequence between two cysteine residues participating in forming a disulfide bridge present in the N-terminal of the entire amino acid sequence of a whole tetanus toxin molecule, splitting said disulfide bridge, and splitting non-covalent bonds between groups on the tetanus toxin molecule;

said tetanus toxin functional fragment antigen having:

- (a) a molecular weight of from 90,000 to 110,000 as measured by an SDS-polyacrylamide gel electrophoresis method;
 (b) an isoelectric point of 7.25 ± 0.5 as measured by an isoelectric focusing method; and
 (c) an immunopotency which is substantially the same as that of a whole tetanus toxin toxoid.
2. The tetanus toxin functional fragment antigen according to claim 1, wherein each of said at least one fragment independently has an N-terminal amino acid sequence selected from the group consisting of the following amino acid sequences (1) to (8):

- (1) KIIPPTNNIRENLYNRTASLTDLGGELCIK,
 (2) IIPPTNNIRENLYNRTASLTDLGGELCIK,
 (3) ENLYNRTASLTDLGGELCIK,
 (4) NLYNRTASLTDLGGELCIK,
 (5) NRTASLTDLGGELCIK,
 (6) TASLTDLGGELCIK,
 (7) SLTDLGGELCIK, and
 (8) GGELCIK .

3. The tetanus toxin functional fragment antigen according to claim 1 or 2, which is stabilized with a fixative.

4. A tetanus vaccine comprising, as an active component, the tetanus toxin functional fragment antigen of any one of claims 1 to 3 in an effective immunogenic amount.
5. A combined vaccine comprising, as one of a plurality of active components, the tetanus toxin functional fragment antigen of any one of claims 1 to 3 in an effective immunogenic amount.
6. A method for producing a tetanus vaccine, which comprises stabilizing a tetanus toxin functional fragment antigen with a fixative,

said tetanus toxin functional fragment antigen comprising at least one fragment which is substantially the same as that obtained by a process comprising the steps of collecting and purifying an extracellular tetanus toxin from a culture filtrate of Clostridium tetani to obtain an extracellular tetanus toxin molecule, splitting a disulfide bridge present in the N-terminal of the entire amino acid sequence of said extracellular tetanus toxin molecule, and splitting non-covalent bonds between groups on the extracellular tetanus toxin molecule;

said tetanus toxin functional fragment antigen having:

(a) a molecular weight of from 90,000 to 110,000 as measured by an SDS-polyacrylamide gel electrophoresis method;

(b) an isoelectric point of 7.25 ± 0.5 as measured by an isoelectric focusing method; and

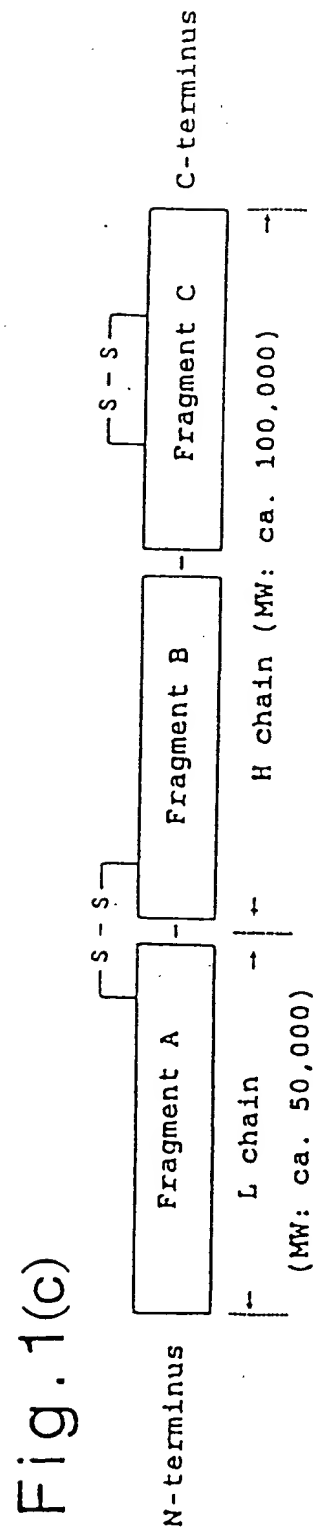
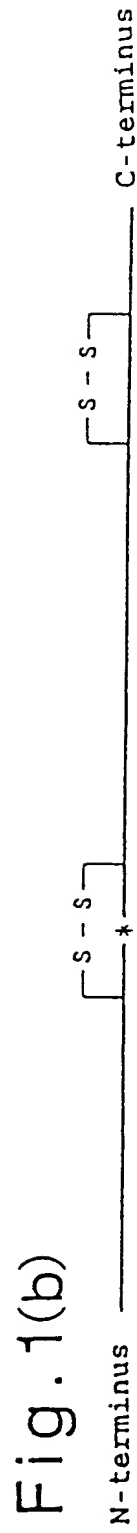
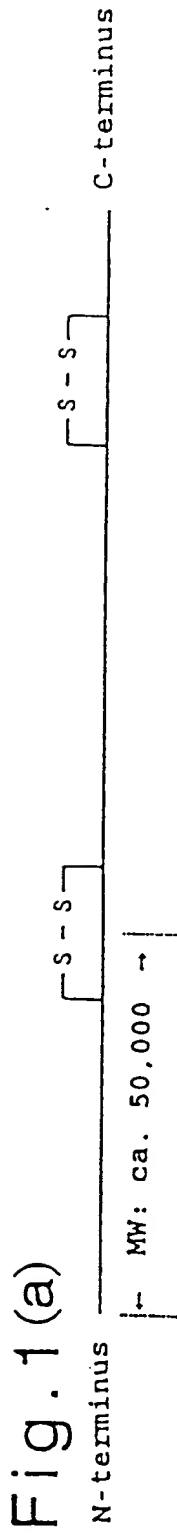
(c) an immunopotency which is substantially the same as that of a whole tetanus toxin toxoid.

7. A method for producing a tetanus toxin functional fragment antigen, comprising:

ligating a DNA coding for the tetanus toxin functional fragment antigen of claim 1 or 2 to a vector;

transforming host cells, exclusive of Clostridium tetani, with said vector; and

expressing said DNA coding for said tetanus toxin functional fragment antigen.



("MW" means molecular weight)

Fig. 2(a)

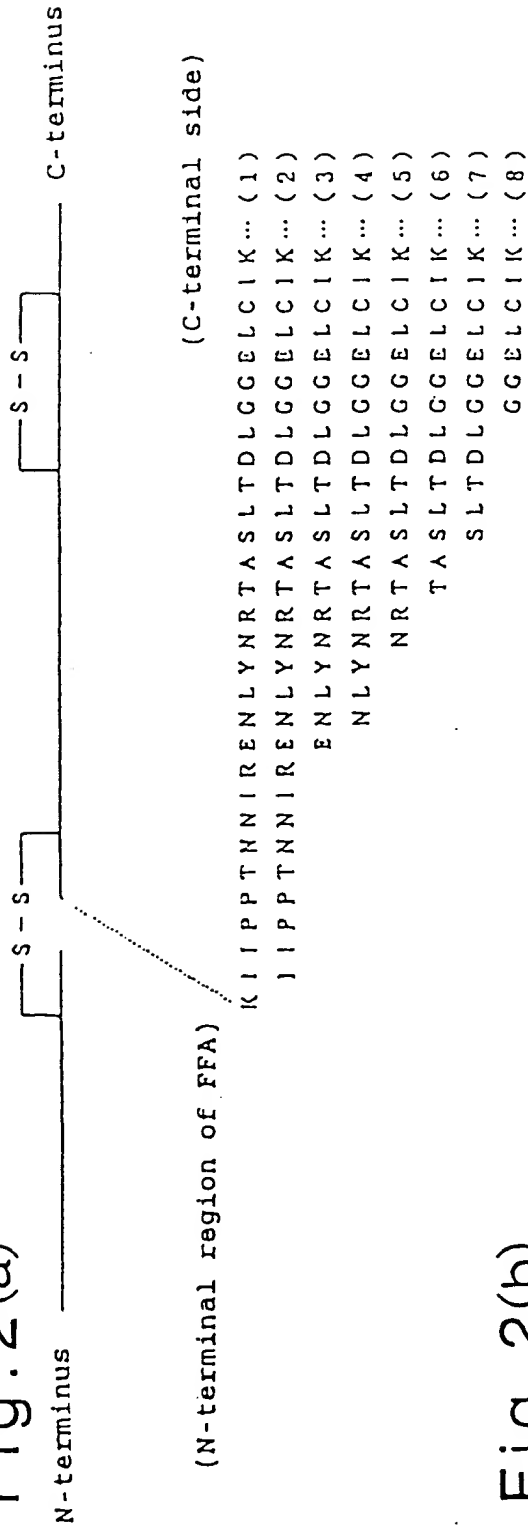
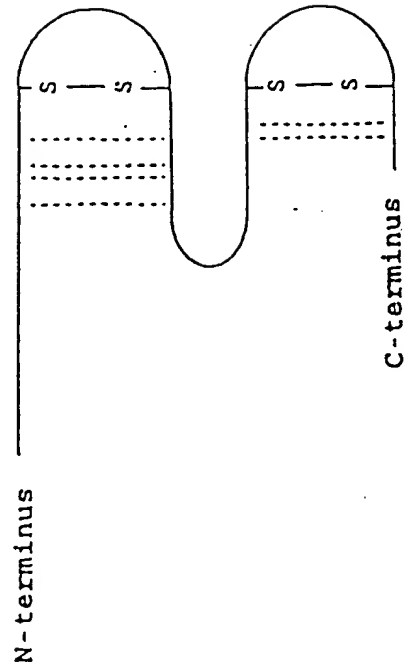


Fig. 2(b)



INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP97/00976

A. CLASSIFICATION OF SUBJECT MATTER Int. Cl ⁶ A61K39/08, C12N15/00, 15/31, C12P21/02 // C07K14/33 According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) Int. Cl ⁶ A61K39/08, C12N15/00, 15/31, C12P21/02 // C07K14/33 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) CAS ONLINE		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	JP, 51-82719, A (Behringwerke AG.), July 20, 1976 (20. 07. 76) & DE, 2457047, A & FR, 2293217, A & US, 4029765, A	1 - 6 7
Y	EISEL, Ulrich et al. "Tetanus toxin: primary structure, expression in E. coli, and homology with botulinum toxins", The EMBO Journal, 1986, Vol. 5, No. 10, pp. 2495-2502	7
Y	JP, 59-2689, A (The Research Foundation for Microbial Diseases of Osaka University), January 9, 1984 (09. 01. 84) & FR, 2528070, A & DE, 3320339, A & US, 4703005, A	7
A	JP, 51-63929, A (Behringwerke AG.), June 2, 1976 (02. 06. 76) & DE, 2448530, A & FR, 2287237, A & US, 4033819, A	1 - 7
<input type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.		
* Special categories of cited documents: - "A" document defining the general state of the art which is not considered to be of particular relevance - "E" earlier document but published on or after the international filing date - "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) - "O" document referring to an oral disclosure, use, exhibition or other means - "P" document published prior to the international filing date but later than the priority date claimed - "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention - "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone - "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combinations being obvious to a person skilled in the art - "A" document member of the same patent family		
Date of the actual completion of the international search May 30, 1997 (30. 05. 97)		Date of mailing of the international search report June 10, 1997 (10. 06. 97)
Name and mailing address of the ISA/ Japanese Patent Office Facsimile No.		Authorized officer Telephone No.

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